

EXPERIMENTAL ASSESSMENT OF CARBACETAM EFFECT ON THE CEREBRAL MITOCHONDRIA IN RATS WITH SCOPOLAMINE-INDUCED ALZHEIMER'S DISEASE

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ABSTRACT

Introduction. One of the pathogenic chains in neurodegenerative diseases, and the Alzheimer's disease especially, is the disorder of cellular energy supply. Investigation of changes in the indices characterizing a functional state of the mitochondria in the cerebral cortex and hippocampus under the influence of carbacetam, a new modulator of GABA-system, is of a certain interest.

The objective of the study was to evaluate the protective effect of carbacetam under conditions of mitochondrial dysfunction of the brain in rats with scopolamine-induced Alzheimer's disease.

Materials and methods. The experiments were conducted on laboratory nonlinear albino male rats with the body weight of 0.18-0.20 kg. To simulate Alzheimer's disease, scopolamine hydrochloride (Sigma, USA) was injected in rats through the peritoneum, in the dose of 1 mg/kg for 27 days. Since the 28th day of the experiment, carbacetam was introduced through the peritoneum in the dose of 5 mg/kg, in 1 mL of 0.9 % NaCl solution, once a day for 14 days.

RÉSUMÉ

Évaluation expérimentale de l'effet du carbacétam sur l'état des mitochondries cérébrales chez les rats avec de la maladie d'Alzheimer induite par la scopolamine

Introduction. L'une des chaînes pathogéniques des maladies neurodégénératives, en particulier la maladie d'Alzheimer, est une violation de l'apport énergétique des cellules. L'étude des changements sur l'évolution des indicateurs qui caractérisent l'état fonctionnel des mitochondries du cortex cérébral et de l'hippocampe sous l'influence d'un nouveau modulateur du système ergonomique GABA présente un certain intérêt.

Le but de l'étude. Évaluer l'effet protecteur du carbacétam dans des conditions de dysfonctionnement cérébral mitochondrial chez des rats atteints de la maladie d'Alzheimer induite par la scopolamine.

Matériaux et méthodes. Les expériences ont été réalisées sur des rats blancs de laboratoire non linéaires, mâles, pesant 0,18-0,20 kg. Le modèle de la maladie d'Alzheimer a été créé par injection intrapéritonéale de scopolamine à une dose de 1 mg / kg pendant 27

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Results. Under conditions of scopolamine-induced Alzheimer's disease, light dispersion decreases and a relative rate of mitochondrial swelling increases in the mitochondrial fraction of the cerebral cortex and hippocampus of rats; free radical oxidation of lipids and proteins increases, and activity of Krebs cycle enzymes decreases – α -ketoglutarate dehydrogenase and succinate dehydrogenase. After carbacetam administration for 14 days, a gradual decrease of light dispersion and relative rate of mitochondrial swelling are found in the mitochondrial fraction of the cerebral cortex and hippocampus. Moreover, the content of products reacting with 2-thiobarbituric acid and protein oxidation modification in the examined structures decreases; the activity of catalase, α -ketoglutarate dehydrogenase, succinate dehydrogenase increases, and superoxide dismutase – only in the cerebral cortex

Conclusions. Reduced intensity of mitochondrial swelling, improvement of the antioxidant system state and energy supply of mitochondria in the cerebral cortex and hippocampus of rats with scopolamine-induced Alzheimer's disease are indicative of mitoprotective properties of carbacetam as a promising neuroprotector.

Keywords: carbacetam, scopolamine-induced Alzheimer's disease, functional state of the mitochondria.

Abbreviations: GABA – gamma-aminobutyric acid, LPO – lipid peroxide oxidation, AP TBA – active products of thiobarbituric acid, CPH – carboxyl phenylhydrazine, SOD – superoxide dismutase, α -KGDH – α -ketoglutarate dehydrogenase, SDH – succinate dehydrogenase.

INTRODUCTION

A regulating role of the brain in the integrating functioning of organs and systems is provided by a concerted action of neurons and glia cells, due to great amount of energy generated by mitochondria. These organelles, contained mostly in the cells of the central nervous system, are known to play a leading role in maintenance of energy metabolism, accordingly to the functional requirements of the brain. Therefore, mitochondria are considered as the most important intracellular structures, determining changes of neurons with pathological and neuro-destructive processes particularly, since they are the first to experience harmful effects¹⁻³. The changes in the permeability of mitochondrial internal membrane are known to be associated with opening of the mitochondrial pore⁴. Under physiological conditions, pore

opening is initiated by a joint action of factors such as free radical and peroxide compounds: active forms of oxygen and nitrogen, respiratory inhibitors and depolarizing agents especially. Their excess, associated with Ca^{2+} -overload, induces an excessive pore opening with pathological processes, the sign of which is mitochondrial swelling⁴. Considering the above, pharmacological correction of the mitochondrial functional state seems to be reasonable. Today, mitochondria attract attention as a target to estimate the efficacy of existing and potential pharmacological neuroprotectors with neurodegenerative diseases.

Résultats. Il a été constaté que dans les conditions du développement de la maladie d'Alzheimer induite par la scopolamine dans la fraction mitochondriale du cortex cérébral et de l'hippocampe des rats, la diffusion de la lumière diminue et le taux relatif du gonflement mitochondrial augmente ; l'oxydation des radicaux libres des lipides et des protéines accroît et l'activité des enzymes du cycle de Krebs décroît – la α -cétoglutarate déshydrogénase et la succinate déshydrogénase. Après l'administration du carbacétam temps de 14 jours dans la fraction mitochondriale du cortex cérébral et de l'hippocampe, il y a une diminution progressive de la diffusion de la lumière et une diminution du taux relatif du gonflement mitochondrial. De plus, la teneur en produits réagissant avec l'acide 2-thiobarbiturique et la modification oxydative des protéines est réduite dans les structures étudiées; l'activité de la catalase, de l' α -cétoglutarate déshydrogénase, de la succinate déshydrogénase augmente et de la superoxyde dismutase uniquement dans le cortex cérébral accroît.

Conclusions. Ainsi, une diminution de l'intensité du gonflement mitochondrial, ainsi qu'une amélioration de l'état du système antioxydant et de l'approvisionnement énergétique des mitochondries du cortex cérébral et de l'hippocampe de la maladie induite par la scopolamine d'Alzheimer, indiquent les propriétés mitoprotectrices du carbacétam, en tant qu'un neuroprotecteur prometteur .

Mots-clés: carbacétam, maladie d'Alzheimer induite par la scopolamine, état fonctionnel mitochondrial.

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Preliminary researches of the Ukrainian scientists, conducted under conditions of the cerebral injury, have found neuroprotective effects of an original derivative of β -carbolinea – carbacetam – a modulator of gamma-aminobutyric acid (GABA) system. It was synthesized at the Department of Chemistry of Active

Compounds, L.M. Lytvynenko Institute of Physical Organic Chemistry and Coal Fuel Chemistry, the National Academy of Science of Ukraine, under the leadership of Doctor of Chemical Sciences S.L. Bogza. We have also found that carbacetam improves anamnestic functions and produces a correcting effect on peroxide oxidation of proteins and lipids of cytosolic fraction in the cerebral cortex and hippocampus of rats with scopolamine-induced Alzheimer's disease^{5,6}. Considering the fact that one of the pathogenic links of neurodegenerative diseases is inhibition of bioenergetic processes in neurons, investigation of changes in the indices characterizing a functional state of mitochondria under the influence of new Ukrainian neuroprotector carbacetam seems to be interesting.

THE OBJECTIVE OF THE STUDY was to evaluate the carbacetam protective effect under conditions of mitochondrial dysfunction of the brain in rats with scopolamine-induced Alzheimer's disease.

MATERIALS AND METHODS

The experiments were conducted on laboratory nonlinear albino male rats, with the body weight of 0.18-0.20 kg, kept under standard vivarium conditions with natural day and night changes, according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (18.03.1986), the Directive of the European Union N° 609 (24.11.1986) and the Order of the Ministry of Health of Ukraine N° 690 (23.09.2009).

To simulate Alzheimer's disease, scopolamine hydrochloride (Sigma, USA) was injected in rats through the peritoneum in the dose of 1 mg/kg once a day during 27 days⁵. On the 28th day of the experiment, a group of rats with Alzheimer's disease (7 rats) began a course of treatment (14 days) with carbacetam introduced through the peritoneum in the dose of 5 mg/kg, in the volume 1 ml of 0.9% NaCl solution per 0.1 kg of the body weight. A solvent was introduced through the peritoneum to the groups of comparison in the same regimen: the control (healthy) rats and rats with simulated pathology (7 rats each).

Euthanasia of rats was conducted under light ether narcosis. The brain was removed cold and washed thoroughly with cool 0.9% NaCl solution. The hippocampus and the cerebral cortex were isolated according to the stereotaxic atlas⁷.

Mitochondrial fraction was isolated by means of the differentiation centrifugation method of homogenates of the structures examined. For this purpose, the cerebral cortex and hippocampus were washed with cool (2-4°C) 0.9 % KCl solution, grinded and homogenized in 10-fold volume of the buffer pH

7.4: sucrose 250 mM, ethylene diamine tetraacetate (EDTA) 1 mM, tris-HCl 10 mM⁸. The homogenate was centrifuged at 700 g, for 10 minutes (4°C), supernatant - at 11 000 g, for 20 minutes (4°C). The sediment was re-suspended in 5 ml of the buffer pH 7.4 (without EDTA) and centrifuged again under similar conditions. The sediment obtained (mitochondrial fraction) was re-suspended in the same buffer and immediately used for investigations.

Swelling of the mitochondria was registered by their ability to extension-contraction and changes of optic density. A relative rate of mitochondrial swelling in the incubation solution was calculated by means of changing E_{520} value⁹. Mitochondrial suspension was placed into the incubation buffer pH 7,4 (mmol/L): sucrose - 150, KCl - 50, KH_2PO_4 - 2, succinate - 1, tris-HCl - 5 (final volume 3 ml). The level of mitochondrial suspension optic density at λ 520 nm during 60 minutes of swelling with the presence of inductor Ca^{2+} (50 $\mu\text{mol/L}$) was registered. Protein concentration in the medium of incubation was 0.4 mg/mL. The change of the mitochondrial swelling level was determined as the difference between the rate of organelle swelling on the 5, 10, 15, 20, 30, 40, 60 minutes relatively the initial level. The mitochondrial suspension in the incubation medium was used as a control with inductor absent with further registration of optic density for 60 minutes. The change of E_{520} values in the incubation medium was used for the calculation of a relative rate of mitochondrial swelling¹⁰, which characterizes changes in the permeability of the organelle internal membrane (massive swelling and depolarization of mitochondria) resulting from mitochondrial pore formation due to overloading of cells with calcium ions.

The state of lipid peroxide oxidation (LPO) in the mitochondria was evaluated by the levels of active products of thiobarbituric acid (AP TBA)¹¹; carbonylation of mitochondrial proteins - by the amount of derivatives of 2,4-dinitrophenylhydrazine with formation of carboxyl phenylhydrazine (CPH) and expressed in nmol of carbonyl derivatives per 1 mg of protein¹². The state of the antioxidant protection system in the mitochondria was evaluated by the activity of superoxide dismutase (SOD) enzymes [EC 1.15.1.1] and catalase [EC 1.11.1.6]¹³. To evaluate energy supply of the mitochondria, the activity of enzymes of α -ketoglutarate dehydrogenase (α -KGDH) [EC 1.2.4.2] and succinate dehydrogenase (SDH) [EC 1.3.5.1] by means of spectrophotometric method was determined¹⁴. The content of protein in the mitochondria was determined by means of Lowry method¹⁵.

The results of the study were statistically processed by means of Student criterion. To confirm reliability of the conclusions Mann-Whitney

nonparametric criterion of comparison was used as well. It demonstrated similar results of calculations made by means of Student criterion concerning p value. Differences were considered statistically valuable with $p \leq 0,05$.

RESULTS AND DISCUSSION

The dynamics of changes in intensity of mitochondria swelling of the examined brain structures is

presented in Fig. 1a, b. In control rats, after incubation of mitochondrial suspension of the cerebral cortex and hippocampus for 60 minutes, the light dispersion decreased by 8% and 9.4%, respectively. In rats with scopolamine-induced Alzheimer's disease, light dispersion of mitochondrial suspension 60 minutes after incubation decreased by 17.1% in the cerebral cortex and by 21.5 % in the hippocampus. After 14 days of carbacetam administration, a gradual decrease of light dispersion, both in the cerebral cortex

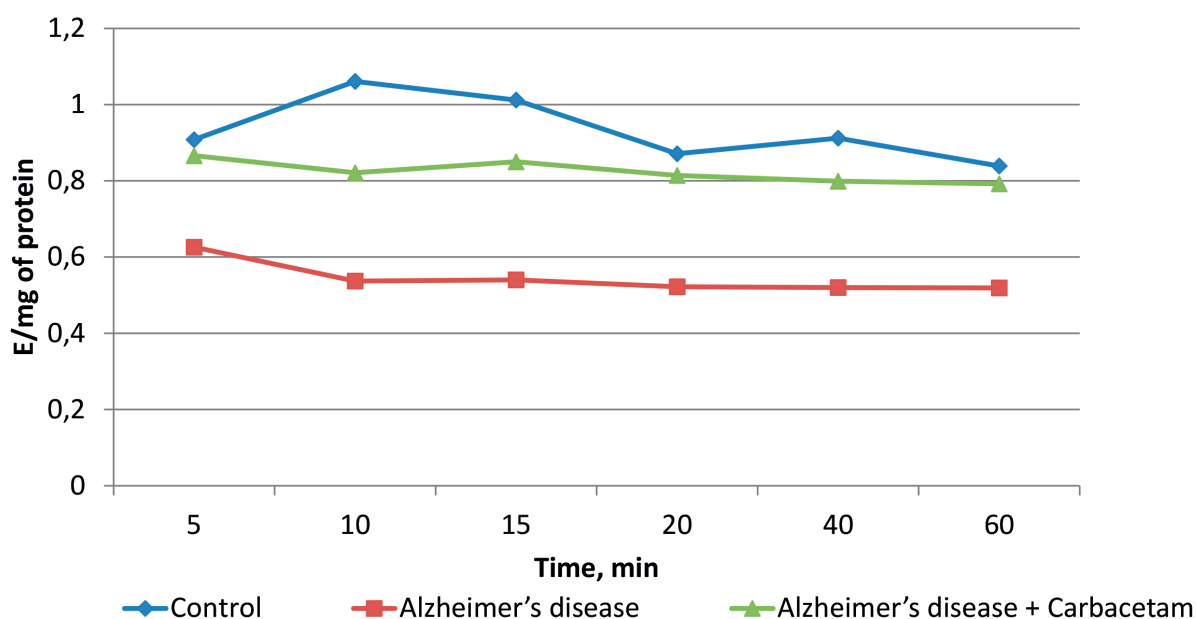


Fig. 1a. Intensity of mitochondrial swelling in the cerebral cortex of rats with scopolamine-induced Alzheimer's disease after carbacetam administration for 14 days in the dose of 5 mg/kg

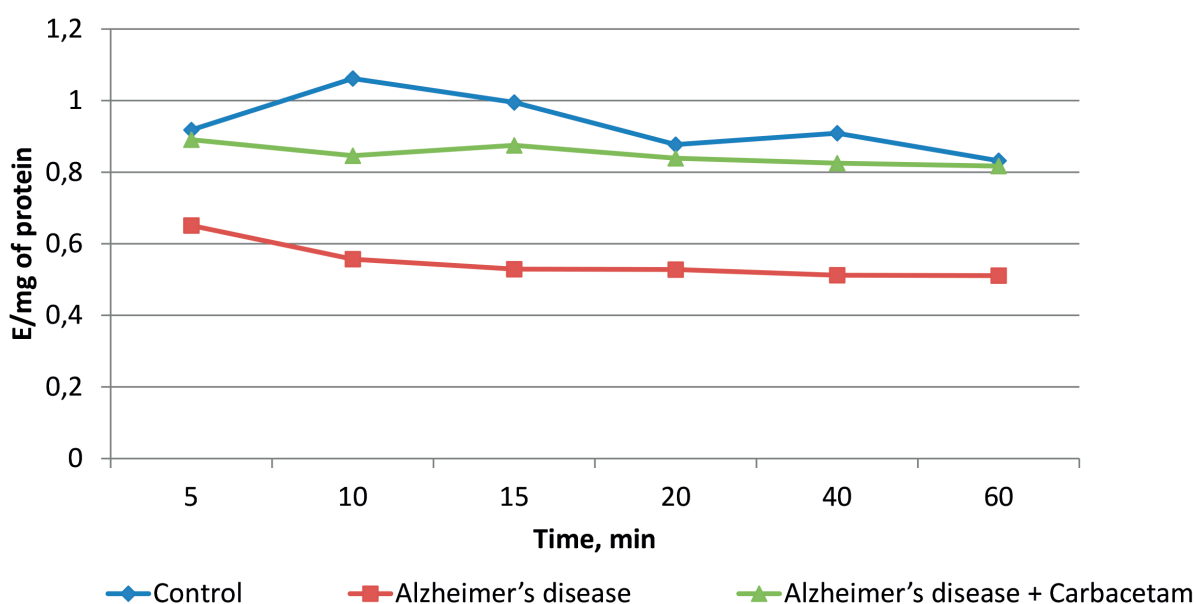


Fig. 1b. Intensity of mitochondrial swelling in the hippocampus of rats with scopolamine-induced Alzheimer's disease after carbacetam administration for 14 days in the dose of 5 mg/kg

and hippocampus of rats with Alzheimer's disease, was observed – 8.5 and 8.3 % respectively.

The results of our investigations showed that a relative rate of mitochondrial swelling in rats with scopolamine-induced Alzheimer's disease increased by 18.8% and 23.5 %, respectively, in the cerebral cortex and hippocampus in comparison with the control group (Fig. 2). It should be noted that after carbacetam administration, a relative rate of mitochondrial swelling decreased in comparison with the data of rats with modelled pathology in both examined structures: 5.3 % – in the cerebral cortex and 9.5 % – in the hippocampus.

Therefore, in rats with scopolamine-induced Alzheimer's disease, the sensitivity of the mitochondrial pore to Ca^{2+} action increases, which might be associated with Ca^{2+} overload. The cause of these processes is the disorder of the membrane permeability for these ions. At the same time, carbacetam administration for 14 days to rats with Alzheimer's disease provoked a decrease of mitochondrial swelling. The main mechanism of carbacetam action might be associated with prevailing intensification of NAD-dependent oxidation, which is one of the ways to increase resistance of the mitochondrial respiratory chain. Moreover, carbacetam is not excluded to modulate Ca^{2+} and K^+ currents. Activation of

mitochondrial ATP-dependent potassium channels is known to result in the decrease of Ca^{2+} load and inhibition of an excessive opening of the mitochondrial pore^{16,17}.

Considering that the mitochondrial pore complex contains numerous amounts of targets and is regulated by endogenous effectors and oxygen active forms particularly, we examined the state of the pro-oxidant-antioxidant system of mitochondria (Table 1). According to the results obtained, the content of AP TBA in these organelles with Alzheimer's disease increased by 1,7 times in the cerebral cortex, and twice as much in the hippocampus, as compared to the data of the control group. CPH content in the mitochondria of both examined structures increased by 1,5 times. This indicates the intensification of the processes of free radical lipid and protein oxidation and damage of the biological membranes. At the same time, functional disorders of the antioxidant protection enzyme systems were observed, which was confirmed by 1.4 and 1.3 times reduced activity of SOD and catalase in the cerebral cortex, and by 1.8 times decrease of catalase in the hippocampus. One of the probable causes of a reduced activity of the examined enzymes can be their oxidation modification under conditions of pathological process development with neuro destruction.

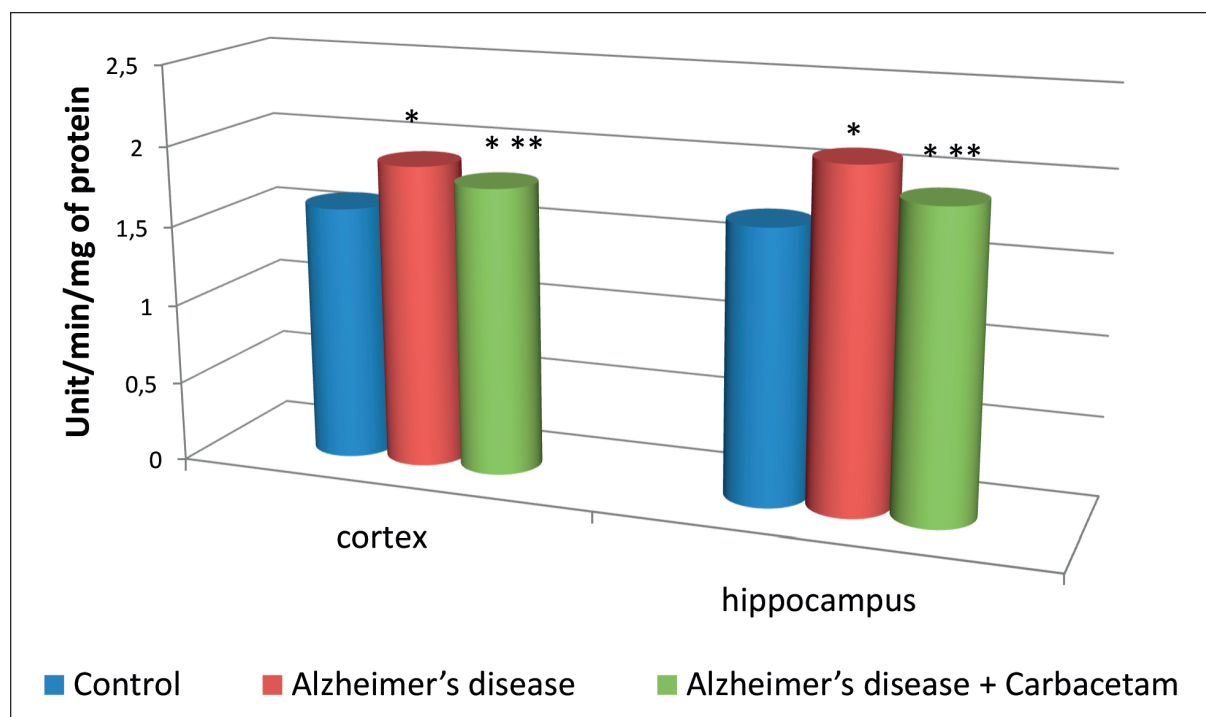


Fig. 2. Relative rate of mitochondrial swelling in the cerebral cortex of rats with scopolamine-induced Alzheimer's disease after carbacetam administration for 14 days in the dose of 5 mg/kg (M±M, n=7)

Notes: * – reliability of differences compared with the control group of rats,

** – reliability of differences compared with the group of rats with Alzheimer's disease.

Table 1. Carbacetam effect on free radical lipid and protein oxidation, energy supply in the mitochondria of the cerebral cortex and hippocampus of rats with scopolamine-induced Alzheimer's disease (M±m, n=7)

Indices	Examined structures cerebral	Control	Alzheimer's disease	Alzheimer's disease + Carbacetam
The content of AP TBA, nmol/mg of protein	cortex	12.8±1.25	21.8±1.50*	16.7±0.88* **
	hippocampus	11.6±0.65	23.3±1.30*	14.9±0.68* **
The content CPH, nmol/mg of protein	cortex	24.7±1.39	36.2±2.62*	26.1±0.79**
	hippocampus	18.3±1.10	26.7±1.21*	19.1±1.11**
The activity of SOD, units/ mg of protein	cortex	0.43±0.027	0.31±0.017*	0.39±0.008**
	hippocampus	0.38±0.045	0.26±0.054	0.37±0.042
The activity of catalase, mcmol H ₂ O ₂ / min of mg of protein	cortex	175.9±10.58	131.2±10.02*	193.6±25.19**
	hippocampus	170.2±10.99	92.6±15.16*	153.9±13.06**
The activity of α- KGDH, nmol/ min of mg of protein	cortex	40.4±2.23	26.1±1.16*	32.2±2.18* **
	hippocampus	43.5±2.24	24.8±1.33*	36.2±2.54**
The activity of SDH nmol min of mg of protein	cortex	6.6±0.57	2.4±0.37*	3.9±0.49* **
	hippocampus	7.3±0.32	3.3±0.31*	4.9±0.56* **

Notes:* – reliability of differences compared with the control group of rats,

** – reliability of differences compared with the group of rats with Alzheimer's disease.

The results showed that development of cerebral pathology was associated with reduced activity of dehydrogenase, which determines efficacy of energy supply of mitochondria. The indices of Krebs cycle energy metabolism decreased in the cerebral mitochondria of rats with scopolamine-induced Alzheimer's disease, in particular: activity of NAD⁺-dependent α-KGDH and FAD⁺-dependent SDH. Thus, in comparison with the indices of the control group, the activity of α- KGDH and SDH in the cerebral cortex decreased by 1.6 and 2.8 times, in the hippocampus – 1.8 and 2.2 times respectively. The results obtained do not contradict the data obtained by other researchers¹. In particular, the decrease of mitochondrial α- KGDH activity is known to correlate with the degree of cognitive disorders with Alzheimer's disease¹⁸.

Further analysis of the results showed that after carbacetam administration in rats with Alzheimer's disease the content of AP TBA in the cerebral cortex decreased by 1.3 times, and it decreased by 1.5 times in the hippocampus, in comparison with the indices in the groups without correction. Moreover, under carbacetam effect, the content of CPH products in both structures decreased on average by 1.4 times, which in general is indicative of inhibition of lipid and protein peroxidation processes.

The comparison of the indices in the groups with carbacetam administration and without treatment determined an increased activity of the antioxidant protection enzymes. Thus, in the cerebral cortex, SOD and catalase activity increased by 1.3 and 1.5 times, respectively. In the hippocampus, the

catalase activity increased only by 1.7 times. It should be noted that it is the hippocampus that is damaged first in Alzheimer's disease¹⁸. At the same time, an increased activity of Krebs cycle enzymes was found: α-KGDH and SDH activity increased by 1.2 and 1.6 times in the cerebral cortex, and in the hippocampus – by 1.6 and 1.5 times, respectively.

Therefore, the results of our study are indicative of the fact that, under conditions of Alzheimer's disease, modelling mitochondrial dysfunction occurs, caused by an increased sensitivity of the mitochondrial pore to Ca²⁺ ions – inductors of its opening, and disorders of pro-oxidant-antioxidant balance in the mitochondria. Carbacetam decreases the intensity of mitochondrial swelling, induces activation of the antioxidant system enzymes and Krebs cycle, reduces the content of lipid and protein peroxidation products, which is indicative of its mitoprotective effects¹⁹.

One of the possible mechanisms of carbacetam action is its modelling effect on GABA system through the receptors of A type, which regulate the permeability of chlorine channels²⁰. This type of receptors are localized in the vascular walls of the brain, whose stimulation promotes vasodilation and improvement of cerebral circulation^{21,22}. Volumetric blood flow and cerebral oxygenation increase correspondingly, which inhibits the intensity of lipid and protein peroxidation processes (decreased content of AP TBA and CPH). At the same time, the activity of the antioxidant protection enzymes (SOD) increases, which neutralizes superoxide anion radical. It is known to be a founder of other active forms

of oxygen and catalase, which destroys such an aggressive form of oxygen as hydrogen peroxide^{23,24}. It enables to suggest a certain antioxidant effect of the examined agent. The data concerning a positive correction effect of carbacetam by means of decreased free radical damage and metabolism improvement, and energy supply of the neurons in the brain of rats with experimental cerebral injury are obtained in several studies²⁵.

In addition to the antioxidant effect, carbacetam promoted to restore activity of enzymes of Krebs cycle (α -KGDH and SDH), supplying respiratory chain of the mitochondria according to reduced equivalents NADN+H⁺ and FADH₂. Therefore, it promotes ATP synthesis by means of oxidation phosphorylation, providing improvement of energy metabolism of the mitochondrial neurons in the cerebral cortex and hippocampus. All the above prevent swelling of the organelles.

The results obtained demonstrate a protective effect of carbacetam under conditions of mitochondrial dysfunction of the brain, which is indicative of improvement of the mitochondrial functional state, the indices of energy metabolism, activity of the antioxidant system in the mitochondria of the cerebral cortex and hippocampus of rats under conditions of scopolamine-induced Alzheimer's disease.

CONCLUSIONS

Under conditions of scopolamine-induced Alzheimer's disease, light dispersion decreases and a relative rate of mitochondrial swelling increases in the mitochondrial fraction of the cerebral cortex and hippocampus of rats; free radical oxidation of lipids and proteins increases, and activity of Krebs cycle enzymes decreases – α -ketoglutarate dehydrogenase and succinate dehydrogenase. After carbacetam administration for 14 days in rats with Alzheimer's disease, a gradual decrease of light dispersion and relative rate of mitochondrial swelling are found in the mitochondrial fraction of the cerebral cortex and hippocampus.

Under carbacetam effect in rats with Alzheimer's disease, the content of products reacting with 2-thio-barbituric acid and protein oxidation modification in the cerebral cortex and hippocampus decreases; in both examined structures, the activity of catalase, α -ketoglutarate dehydrogenase, succinate dehydrogenase increases, and superoxide dismutase – only in the cerebral cortex.

Reduced intensity of mitochondrial swelling, improvement of the antioxidant system state and energy supply of mitochondria in the cerebral cortex and hippocampus of rats with scopolamine-induced

Alzheimer's disease are indicative of mitoprotective properties of a promising neuroprotector, carbacetam.

Author Contributions:

Conceptualization, O.G.K. and N.D.F.; *methodology*, I.M.Y.; *software*, T.I.K.; *validation*, O.G.K. and T.I.K.; *formal analysis*, O.G.K.; *investigation*, T.I.K.; *resources*, Y.M.V.; *data curation*, Y.M.V. and T.I.H.; *writing – original draft preparation*, O.G.K.; *writing – review and editing*, O.G.K., N.D.F., I.M.Y.; *visualization*, O.G.K. and I.M.Y.; *supervision*, T.I.H.; *project administration*, O.G.K. All the authors have read and agreed with the final version of the article.

Compliance with Ethics Requirements:

„The authors declare no conflict of interest regarding this article“

„The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law.“

„All institutional and national guidelines for the care and use of laboratory animals were followed“

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