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EXPERIMENTAL ARTICLES

The Molecular and Ultrastructural Aspects of the Formation of Mitochondrial Dysfunction in the Modeling of Chronic Cerebral Ischemia: The Mitoprotective Effects of Angiolin

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Abstract—Modeling of cerebral circulation disorder by bilateral occlusion of carotids was accompanied by formation of severe neurological symptoms and their preservation for up to 18 days of the experiment. We found a significant decrease in the level of the HSP_{70} heat shock protein and the formation of persistent mitochondrial dysfunction. Administration of Angiolin to experimental animals resulted in activation of the expression of the HSP_{70} gene in neurons and ultrastructural improvement of mitochondria and neurons of CA1 hippocampal zone. The substantial mitoprotective activity of Angiolin is based on its antioxidant activity and the ability to positively influence the activity of anaerobic glycolysis, thus stabilizing the cellular energy metabolism under conditions of ischemic injury.

Keywords: cerebral circulation disorder, heat shock proteins, mitochondrial dysfunctions, mitoprotective effect, mitoptosis, Angiolin

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INTRODUCTION

Cerebral pathologies are in the third place according to their abundance and mortality in the populations of industrially developed countries and not only lead to a decrease in lifespan but also limit a person's social activities because of developing cognitive deficit [1]. Cerebral diseases of destructive and degenerative origins are associated with the disruption in the mitochondrial redox chain, energy exchange, and ionic cell homeostasis and the development of glutamate excitotoxicity, nitrosative and oxidative stress, and cell death. In modern medicine, the doctrine of polysystemic disorders of cellular energy exchange, so-called mitochondrial pathology or mitochondrial dysfunction, is taking an increasingly significant position [2]. Analysis of endogenous protection mechanisms of neurons against mitochondrial dysfunction and development of new effective drugs that target mitochondria are important tasks of modern pharmacology [3]. At present, in order to correct mitochondrial dysfunction, attempts to use energotrophic drugs are made, viz., coenzyme Q10, carnitine, *B* vitamins, and derivatives of succinic acid [4]. However, the rational basis for their use is poorly developed; effective approaches are often used insufficiently and ineffective approaches are overestimated, drugs are used chaotically without sufficient knowledge of their capacities and features, without planning an appropriate treatment strategy. In addition, in already-developed mitochondrial dysfunction and initiated apoptotic processes, these drugs are inefficient and are not able to participate in the regulation of the fine chains of energy metabolism, where they play the role of intermediates. There is also another aspect of the correction of mitochondrial dysfunction, viz., the use of thiol antioxidants that compete with SH groups of the cysteine-dependent site of the inner mitochondrial membrane protein (ATP/ADP antiporter) for ROS and peroxynitrite and form strong complexes with the latter [5]. This prevents mitochondrial-pore opening under conditions of oxidative and nitrosative stress. All of these factors were the grounds for the creation at the Farmatron NPO (president, prof. I.A. Mazur) of a new metabolitrophic endothelioprotector with an original structure, viz., (S)-2,6-diaminohexanic acid 3-methyl-1,2,3-triazolyl-5-thioacetate, Angiolin, which has anti-ischemic, cardioprotective, neuroprotective, and antioxidant characteristics [6]. Taking the subcellular-molecular-biochemical mechanisms of the development of mitochondrial dysfunction in ischemia and the pharmacological properties of Angi-

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olin into account, the purpose of this work was to study the influence of Angiolin on the ultrastructure of CA1 hippocampal zone neurons and heat-shock proteins under conditions of chronic cerebral ischemia.

MATERIALS AND METHODS

The experiments were performed with white wild-type rats with a 180–200 g weight of both sexes that were acquired from the farm of the governmental Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine. All manipulations were conducted according to the regulations on the use of animals in biomedical experiments (Strasbourg, 1986, with changes made in 1998), which were reconciled with the regulations of the European Convention for the Protection of Vertebrate Animals that Are Used for Experimental and Other Scientific Purposes. The protocols of the experimental studies and their results were approved by the decision of the Commission on Bioethics of ZSMU (Protocol no. 33 from October 26, 2013).

The model of chronic cerebral-circulation disorder (CCD) was induced by bilateral occlusion of common carotid arteries. Taking the high mortality in this experimental model into account, the number of operated animals was such that by 18 days there would be ten animals in each group. The operation was performed under thiopental anesthesia (40 mg/kg). The studied drugs were administrated for 18 days in the form of aqueous solutions intragastrically using a metal probe immediately after the recovery of animals after anesthesia: Angiolin (tablets produced by the Farmatron NPO (Ukraine)) at a dose of 100 mg/kg and mildronate (capsules produced by the JSC Grindeks (Latvia)) at a dose of 250 mg/kg. Thus, four groups of experimental animals were formed: sham-operated animals ($n = 10$), animals with cerebral-circulation disorder, which was a control group ($n = 10$), animals with CCD that received Angiolin ($n = 10$), and animals with CCD that were treated with mildronate ($n = 10$). By the end of the experiment, according to the study protocol, the animals were anesthetized with sodium thiopental (40 mg/kg), the skull was opened, and the brain was taken [7].

Pharmacological methods included the estimation of neurological deficit by the C.P. McGraw Stroke Index. The severity of the state was estimated according to the sum of the corresponding points. We recorded the quantity of animals with mild symptoms of up to 2.5 points (sluggishness of movements, weakness of limbs, unilateral hemiptosis, tremor, and circus movements) and severe manifestations of neurological disorders (from 3 to 10 points), viz., pareses of limbs, paralysis of lower limbs, and a lateral posture [8].

The brain tissues were homogenized in cold isotonic saline (0.15 M KCl) at 4°C with a SilentCrusher S homogenizer (Germany) with a tissue to saline ratio of 1 : 40. The obtained suspension was centrifuged for 7 min at 700 *g* in an Eppendorf Centrifuge 5810R. The obtained precipitate was discarded and the supernatant liquid was repeatedly centrifuged at 11000 *g* in a Sigma 3-30k Refrigerated Centrifuge at 4°C. The supernatant represented the cytosolic fraction and the obtained sediment contained the mitochondrial suspension.

The HSP_{70} concentration in the cytoplasmic and mitochondrial fractions was estimated by Western blot analysis in a 10% polyacrylamide gel (PAAG). The protein was transferred to a nitrocellulose membrane at a voltage of 100 V and a current of 0.35 A for 1 h. After the transfer, the membrane was placed into a blocking buffer that contained a 1% bovine serum albumin solution (Sigma, United States) for 20 h. After washing in 0.1 phosphate buffer solution the membrane was placed in a solution of primary anti-HSP_{70} antibodies (1 : 500) (Santa Cruz Biotechnology) and incubated for 2 h at room temperature. We then performed a repeated washing in 0.1 M phosphate buffer, placed the membrane in a solution of secondary antibodies (1 : 1000) (biotin-conjugated anti-mouse IgG, Sigma, United States) and incubated it for 2 h. For visualization, the membrane was treated with AEC: one tablet of 3-amino-9-ethyl carbazole (Sigma, United States) was dissolved in 2.4 mL DMF, which contained 47.5 mL of 0.05 M acetate buffer, pH 5.0, and 25 μL of 30% H_2O_2. The membrane was incubated in the substrate mixture for 5–10 min. A red insoluble precipitate characterizes the antigen–antibody complex. The membrane was washed in distilled water several times. The stripes were dried between sheets of filter paper under a cold air flow. The HSP_{70} level was measured by densitometry using Adobe Photoshop software. The results were expressed in arbitrary units per gram of protein. The protein level was estimated by direct spectrophotometry at a wavelength of $\lambda = 280$ nm.

For the transmission electron microscopy study, the brain tissue (the CA1 zone of the hippocampus) was fixed with a 3% glutaraldehyde solution in a buffer at pH 7.4 for 2 hours at 4°C; fixation was then finished with a 1% OsO_4 solution in the buffer for 1.5 h and dehydrated in solutions of alcohols of increasing concentration (70% alcohol was saturated with uranyl acetate). The tissue samples were embedded into Epon-812 epoxy resin, which was diluted with acetone by 3, 2, and 1.5 times, respectively; each time we left the preparations for impregnation in resin overnight at room temperature. At the last stage the preparations were placed in undiluted resin at 37°C for the night with the following polymerization of Epon-812 at 60°C to the solid glassy state. Serial ultrathin sections were prepared on a LKB-III ultramicrotome and stained with lead using Reynolds' method. Seven preparations were made from each tissue sample of

Table 1. The influence of Angiolin and mildronate on neurological status and HSP_{70} level in animal cerebral tissues of animals with CCD on the 18th day of the experiment (M ± m)

Animal group	HSP_{70}, arb.un./g protein		Average score by McGrow's scale
	Mitochondrial fraction	Cytosolic fraction	
Sham operated ($n = 10$)	7.20 ± 0.47	15.41 ± 0.60	–
CCD (control) ($n = 10$)	2.10 ± 0.46	6.95 ± 0.47	6.6 ± 0.91
CCD + mildronate, 250 mg/kg ($n = 10$)	2.13 ± 0.33	7.35 ± 0.68	5.65 ± 1.13
CCD + Angiolin, 100 mg/kg ($n = 10$)	8.66 ± 0.74#&	22.73 ± 2.31#&	2.5 ± 0.53#&

# $p < 0.001$ as compared to the control group; & $p < 0.001$ as compared to mildronate-treated group.

one animal. The obtained preparations were viewed and photographed in an electron microscope.

The results of the study were processed using Statistica software for Windows 6.0 (StatSoft, Inc., United States), SPSS 16.0, and Microsoft Excel 2003 using the Student's *t* test [9]

RESULTS AND DISCUSSION

Modeling of chronic cerebral ischemia via disturbance of cerebral circulation resulted in persistent neurological disorders in the animals by the 18th day of experiment. The average McGrow rate was 6.6 ± 0.91 in the control animals. (Table 1). Using Western blotting and electron microscopy, we detected the signs of mitochondrial dysfunction in animals from the control group. Thus, as a result of the studies we conducted, we detected a decrease in the HSP_{70} level in the brain of experimental animals (Table 1). In the control group of animals a 2.2- and 3.2-fold HSP_{70} decrease was observed in cytosolic and mitochondrial cerebral homogenate fractions, respectively by the 18th day of the experiment, as compared with the similar indices for sham-operated animals. It is known that the level of endogenous reduced glutathione may regulate heat-shock-protein expression in the cell [10]. Recently, it was shown that heat shock proteins have a protective role in cerebral ischemia, which is accompanied by intensification of free-radical oxidation, a shift in the thiol–disulphide equilibrium, development of nitrosative stress, and glutamate excitotoxicity [11]. Angiolin administration promoted normalization of this index, which suggests that it has protective properties and the ability to prevent inhibition of HSP_{70} expression in neurons. After a course of Angiolin, the HSP_{70} concentration increased by factors of 3.27 and 4.12 in the cytosol and mitochondria of the brain homogenate, as compared to control indices. The concentration in the brain of Angiolin-treated animals was higher than in the intact group by 47.5 and 20.3% in the cytosolic and mitochondrial fractions, respectively. These data suggest that HSP_{70} expression is activated in neurons in the presence of Angiolin. Since this protein is related to heat shock family proteins and possesses chaperone activity, an increase in its level plays a significant role in the normalization of cell vital activity and the prevention of the development of apoptosis and necrosis under conditions of CCD [12].

HSP family proteins are induced in all cells of living organism as a response to the actions of numerous stress factors, such as heat shock, hypoxia, ischemia, metabolic disorders, virus infection, and the effects of pharmacological agents. The genes for these proteins are activated not only in stress conditions but during the main processes of cellular life activities, proliferation, differentiation and apoptosis [13]. Recently, data have been published on the regulatory action of HSP_{70} on the phenomena of mitochondrial dysfunction, which develops in ischemic brain injury [14]. A number of studies show that HSP_{70} in vitro can prevent aggregation of oxidatively damaged citrate synthase, glutathione S-transferase, glutathione reductase, superoxide dismutase, lactate dehydrogenase, and malate dehydrogenase and can regulate the thiol–disulphide equilibrium [15].

In addition, one of the main functions of HSP_{70} is induction and prolongation of the lifetime of the HIF-1α stable form, which engages further adaptive reactions in the cell [16]. An HSP_{70} deficit is one of the main causes of mitochondrial dysfunction with all of its ensuing consequences for cellular activities. We believe that in the brain under conditions of ischemia, an HSP_{70} deficit is associated with overproduction of reactive oxygen species (ROSs) and cytotoxic forms of nitric oxide, which leads under conditions of reduced glutathione deficit not only to modification (reversible and irreversible) of macromolecules, including HSP_{70} itself, but also to a decrease in the expression of genes that encode synthesis of the latter. A number of studies have shown the role of nitric oxide derivatives in the inhibition of gene activity and a decrease in the levels of different transcription factors [17]. Apparently, an excess of such forms of nitric oxide as peroxynitrite and nitrosonium ion first nitrolize thiol-redox-dependent sites of these genes and then, with an increase in concentration, oxidize them. Via the stabilization of oxidatively damaged molecules, HSP_{70} prevents mitochondrial-pore opening, thereby blocking cytochrome *C* release from mitochondria, thus displaying a direct anti-apoptotic action. In addition, heat shock proteins have been shown to have antiapoptotic action, which is determined by the stimulation of the expression of the antiapoptotic protein Bcl-2 [18].

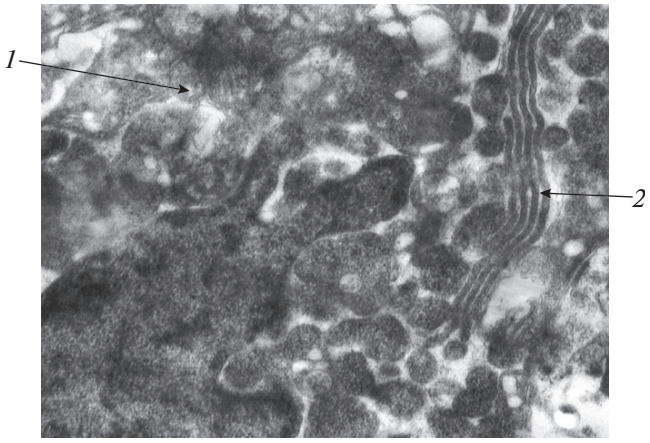

Fig. 1. The sham-operated animal group. Fragment of hippocampal neurocyte of rat. Mitochondria with density-varying cristae and matrix (1). Multiple elements of the granular endoplasmic reticulum. The developed Golgi complex (2). The electron diffraction pattern. ×25000.

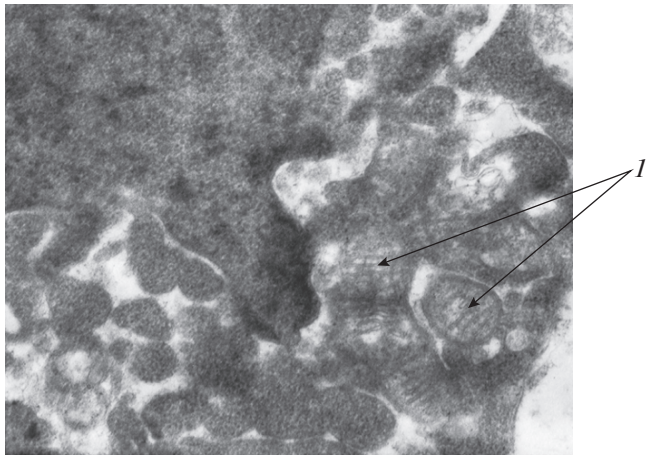

Fig. 2. The sham-operated group. The paranuclear zone of hippocampal neurocyte of rat. The nucleus with moderately active nucleoplasm. Active large mitochondria with signs of intermitochondrial contacts (1). Multiple elements of the granular endoplasmic reticulum. The electron diffraction pattern. ×25000.

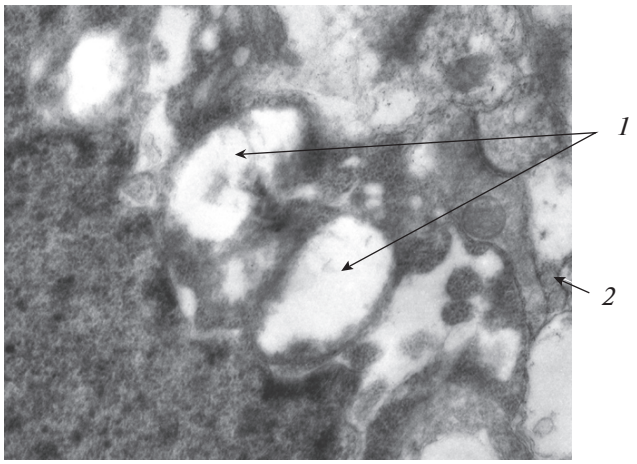

Fig. 3. The untreated CCD animal group on the 18th day (control). The perikaryon area of the rat hippocampus neurocyte. Vacuolar-lytic damage of the mitochondria and endoplasmic reticulum (1). Individual micromitochondria (2). The electron diffraction pattern. ×30000.

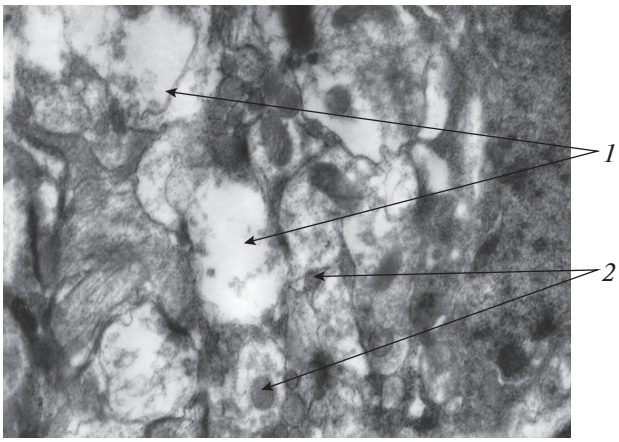

Fig. 4. The untreated CCD animal group on the 18th day (control). A fragment of the hippocampal neurocyte of rat. Vacuolar-lytic damage of the perikaryon. Massive cristolysis of mitochondria (1). Electron-dense inclusions in a compound of multiple mitochondria (2). The destruction of membranes of the granular endoplasmic reticulum. The electron diffraction pattern. ×50000.

Electron microscopy of the hippocampal CA1 zone of animals with CCD on the 18th day of the experiment revealed disturbances in neuronal ultrastructure with mosaic-like character. We observed a part of neurocytes with signs of necrotic and nercobiotic alterations, the majority of cells had nuclei with signs of abruptly and moderately limited functional activity, and fragmented nucleolemma (Figs. 1–4). In perykarion, vacuolar-lytic organelle damage occur (Fig. 3). In neurons with chromatolysis phenomena, there was swelling and vacuolization of lamellar apparatus cisternae, often with the deformation of the cisternae and their fragmentation, an increased number of lysosomes at different stages of maturity, multivesicular corpuscles, and small osmiophilic inclusions. In the endoplasmic reticulum, massive formation of vacuole-like cisternae occurred.

Mitochondrial structure in neurocytes with moderate damage had vacuole-lytic disturbances. The majority of mitochondria increased in size, had a fragmented outer membrane, destroyed cristae, and the electron-transparent matrix. Sometimes we found giant mitochondria with abrupt matrix edema. An insignificant part of the mitochondria had relatively preserved cristae and were small. The presence of mitochondria with a heteromorphic structure was typ-

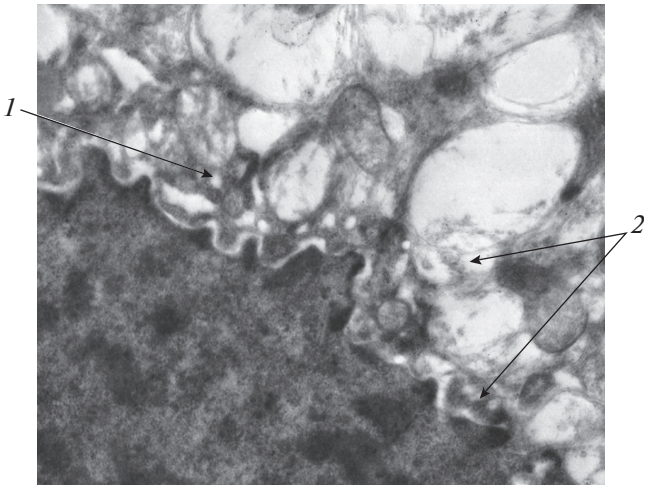

Fig. 5. The CCD animal group treated with Angiolin for 18 days (100 mg/kg). A fragment of a rat hippocampal neurocyte. Mitochondrial-type apoptosis at the induction phase. The destabilization of mitochondrial membranes (1). Partial cristolysis and the irregular electron density of the matrix (2). Coarsely dispersed damage of the nucleoplasm. The electron diffraction pattern. × 30000

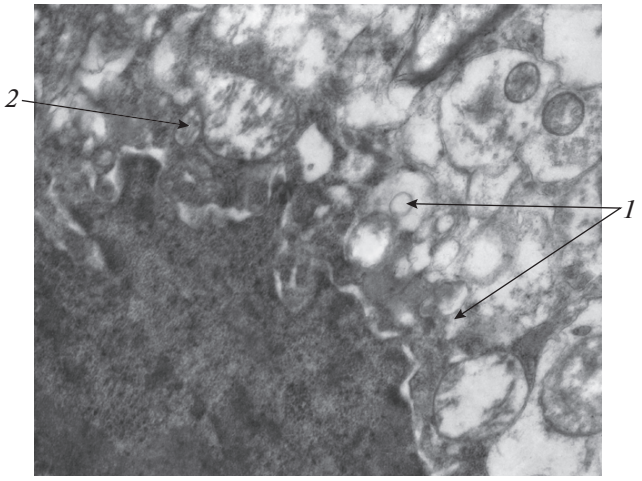

Fig. 6. The CCD animal group that was treated with Angiolin for 18 days (100 mg/kg). The vacuolar type of perikaryon damage (1). The active nucleoplasm. Moderate cristolysis of mitochondria (2). Partial destruction of membranes of the granular endoplasmic reticulum. The electron diffraction pattern. ×30000

ical: part of them contained stable membranes in the cristae, another part contained electron-dense inclusions and a dense matrix.

The disruption of mitochondrial ultrastructure in neurons was observed in a significant part of the damaged neurons, which was characterized by an increase in the absolute quantity of damaged mitochondria by more than 12 times as compared to the intact animal group (Table 2).

A course of Angiolin tablets for 18 days after occlusion of the common carotid arteries resulted in improvement of the ultrastructure of CA1 hippocampal neurons. Thus, structural disruptions in the neurons in this animal group were more diverse as compared to the control group. In addition, we found individual neurons with signs of necrobiotic alterations and a large number of neurocytes with signs of relative adaptation, and neuronal apoptosis figures. Mainly, it is the initial stages of apoptotic processes that develop by the mitochondrial type. In these cells, coarsely dispersed damage of the nucleoplasm with partial preservation of nuclear activity was observed. Lysosomes were not numerous (Fig. 5). In the perikaryon, a moderate disorganization of the granular reticulum occurred with swelling of cisternae and the formation of vacuole-like cisternae. Mitochondria had areas of defragmentation of outer membranes, partially destroyed cristae, and an irregular electron density of the matrix. Occasionally, mitochondria with a preserved cristae structure occurred (Fig. 6).

The majority of the neurocytes with moderate vacuolar disruption of the perikaryon contained nuclei with active nucleoplasm or with signs of moderately limited functional activity. The nucleolemma was stable along its entire length without fragmentation. Chromatolysis, swelling, and vacuolization of cisternae of the lamellar apparatus and endoplasmic reticulum were not pronounced.

Mitochondria were presented by structurally different organelles. Expanded mitochondria with vacuolar damage were found without signs of destruction of the outer membranes, moderate cristolysis, and the electron-light matrix. Giant mitochondria were absent. Part of the mitochondria had relatively stable cristae, were small, and had a moderately dense matrix. A number of mitochondria did not differ significantly from the control group but all of them had

Table 2. The influence of Angiolin and mildronate on the state of mitochondria from the hippocampal CA1 area from the brain of animals with CCD on the 18th day of the experiment (M ± m)

Animal group	Number of damaged mitochondria (absolute value)	Percentage of damaged mitochondria, %
Sham operated (n = 10)	7.3 ± 2.21	8.5%
CCD (control) (n = 10)	91.3 ± 5.41	97.4%
CCD + mildronate, 250 mg/kg (n = 10)	80.1 ± 7.84	95.5%
CCD + Angiolin, 100 mg/kg (n = 10)	52.1 ± 3.41#&	48.2%#&

# $p < 0.001$ as compared to the control group; & $p < 0.001$ as compared to the mildronate-treated group.

stable outer membranes and cristae and a homogenous matrix without electron-dense inclusions.

The significant decrease in the total number of damaged mitochondria in the brain of experimentally treated animals was noticeable (Table 2). Thus, Angiolin administration resulted in a decrease in the number of damaged mitochondria by 42.9% as compared with the control animals. The ultrastructure of neurons of the CA1 zone in animals that received mildronate for 18 days after occlusion of both carotids did not differ from the ultrastructure of untreated animal group. The mitoprotective effects of Angiolin exceeded those of mildronate with statistical significance.

The effectiveness of Angiolin is based on its ability to positively influence the activity of anaerobic homeostasis by preserving energy production in the tricarboxylic area, affecting the activation of the dicarboxylic area and thus stabilizing cellular energy metabolism under conditions of ischemic damage [19]. The lysine residue in the Angiolin molecule binds with methionine resulting in the formation of the carnitine molecule. Carnitine plays the role of a "shuttle" in acetyl-CoA formation from fatty acids. Because the activation and oxidation of fatty acid are spatially separated in the cell, there is a mechanism for the transition of long-chain fatty acids across the inner mitochondrial membrane into the mitochondrial matrix accompanied by carnitine and a special enzyme system. This system reversibly transfers an acyl group on the outer side of the membrane from CoA to carnitine, while on the inner side it is transferred from carnitine to intramitochondrial CoA. It is also known that a carnitine-induced increase in mitochondrial metabolism can prevent the formation of peroxynitrite and other free radicals under conditions of hypoxia [20]

The lysine residue in the brain tissues also metabolizes into pipecolic acid, which increases the affinity of the GABA-benzodiazepine receptor complex. This effect of lysine is of particular interest for the condition of glutamate excitotoxicity, which occurs in hypoxia and results in an increase in the intracellular Ca^{2+} concentration, NO-synthase activation, intensive formation of NO and peroxynitrite ($ONOO^-$), which causes cell death. By increasing the affinity of GABA receptors pipecolic acid decreases the hyperexcitability of glutamate receptors and reduces the release of excitatory amino acids (glutamate and aspartate), thus neutralizing the manifestations of glutamate excitotoxicity [21].

CONCLUSIONS

The significant mitoprotective activity of Angiolin is determined by its antioxidant properties, because of which the drug increases the expression of an endogenic neuroprotector, viz., heat shock protein (HSP_{70}) which has with chaperone activity and plays an important role in preserving the ultrastructure of neuronal mitochondria and in their protection from oxidative stress, as well as in the prevention of the development of mitoptosis and the formation of mitochondrial dysfunction [19].

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