

# **Pharmacological Analysis of Neuroprotective Action of Methylprednisolone with Citicoline in Conditions of Experimental Allergic Encephalomyelitis**

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## **Abstract**

The paper is devoted to studying the neuroprotective effects of the integrated use of methylprednisolone with citicoline on the processes of energy providing of the mitochondria of neurons of the cerebral cortex and histomorphometric indices in experimental allergic encephalomyelitis. Cooperative administration of the drugs has a significant effect on the mitochondrial dysfunction (reduction in speed of opening of mitochondrial pores, increasing the charge of the inner membrane) and energy metabolism of the brain (increased levels of ATP and ADP, reduced lactate, increase pyruvate and isocitrate). The combined use of methylprednisolone with citicoline limits unproductive activity of anaerobic glycolysis and increases the aerobic reaction.

**Keywords:** experimental allergic encephalomyelitis, citicoline, methylprednisolone, mitochondria, neurons

Multiple sclerosis (MS) is a multifactorial, autoimmune, chronic inflammatory demyelinating disease of the central nervous system (CNS). It is generally accepted that diffuse white and gray matter of the CNS in MS patients, leading to the development of atrophy of the brain and spinal cord. Severity of neurological symptoms in MS is largely related to the overall brain atrophy, manifested as a decrease in volume of brain parenchyma, and increased ventricular and subarachnoid spaces. The average age of debut of the disease is 29 years, the ratio of female and male cases are usually close to 3: 1 [10]. Disability in MS is primarily associated with impaired motor function, visual impairment, and coordination of pelvic functions. MS - the second most common cause of disability among young people is not only socially but also economically significant disease [5, 16]. Until recently it was believed that a violation of the conducting function of axons in MS occurs only as a result of multifocal lesions of the myelin sheath [17]. However, more recent studies have shown that neurodegeneration (inflammatory damage to gray and white matter of the brain) occurs in the early stages of MS, and plays a large role in the formation of irreversible neurological deficit [4, 9, 11]. Moreover, a clear correlation between the degree of disability in MS data with degenerative brain changes, while the magnetic resonance imaging pattern of inflammatory changes can significantly dissociate with the clinical picture [7]. Data on the primary pathogenesis of neurodegeneration in MS are very few, remain unclear underlying causes and mechanisms of development. These studies are available in clinical practice, not possible to estimate the contribution of neurodegenerative changes in neurological deficit of individual patient, the disease and to predict the effectiveness of therapy. The aim is to investigate the effect of co-administration of methylprednisolone and citicoline to the processes of energy providing of the mitochondria of neurons of the cerebral cortex and histomorphometric parameters of its formation in experimental allergic encephalomyelitis.

## **Methods**

Prior to the implementation of the Commission's work on bioethics upcoming research protocol was approved. According to the requirements of GLP and the European Convention for the Protection of Vertebrate Animals used for experimental and other purposes agreed by all the procedures related to the maintenance of the animals, the humane treatment of them and their use in experiments.

The experimental animals were kept in standard conditions with a light regime of day - night 12 hour / 12 hours at an air temperature of 20 - 22°C with free access to food and water. Experimental allergic encephalomyelitis (EAE) was induced by a single subcutaneous inoculation of encephalitogenic mixture (EGM) in complete Freund's adjuvant (CFA) at the rate of 100 mg of spinal cord homogenate homologous; 0.2 ml CFA (Mycobacterium killed content of 5 mg / ml) and 0.2 ml saline per animal. EGM was injected into the base of the tail under light ether anesthesia in a volume of 0.4 ml of [8].

Brain experimental white rats were used for biochemical studies. Rapidly removed from the brain of blood, separated from the meninges investigated pieces were placed in liquid nitrogen. Then ground in liquid nitrogen to a powder and homogenized in 10 times the volume of the medium at (2 °C) containing (in mmol): sucrose - 250-HCl-Tris buffer - 20 -1 EDTA (pH 7.4). When the temperature (+ 4 °C) by differential centrifugation at refrigerated centrifuge isolated mitochondrial fraction. To clean the mitochondrial fraction of large cell fragments previously conducted centrifugation for 7 minutes at 1000 g, the supernatant was then recentrifuged for 20 minutes at 17000 g. The supernatant was decanted and stored at -800 S. Mitochondrial pellet was resuspended in isolation medium containing bovine serum albumin (0.5 mg / ml) and again precipitated for 10 minutes at 17,000 g. The mitochondria were suspended in isolation medium suspension contained 40-60 mg protein / ml. For long term storage mitochondria is frozen at -80 °C. To determine the speed potential of the inner mitochondrial membrane and opening of mitochondrial pores used suspension 0.5-1.0 mg protein / ml.

Energy metabolism was evaluated by content of adenine nucleotides - ATP, ADP and AMP, and the content of lactate, malate, pyruvate, and aspartate isocitrate. The content of ATP, ADP and AMP, and aspartat performed by flash chromatography followed by UV-spectrometry. Quantitative determination of the content was carried out by the method of pyruvate Tsoha-Lamprecht from the decrease of NADH. Quantitative determination was performed according to the method of Hohorst from the increase in NADH. Determination of isocitrate concentration was performed by the method of Siebert from the increase in NADPH. Determination of lactate was performed according to the method Hohorst from the increase in NADPH.

The development was characterized by the magnitude of the mitochondrial inner membrane of mitochondria potetsiala and the degree of swelling of mitochondria. The potential generated at the inner mitochondrial membrane, was recorded on a spectrophotometer, a two-wave mode (511 - 533 nm) with Safranin O. as a voltage-dependent probe (18 uM). Measurements were performed in 10x10 mm glass cell with a working volume of 2 ml. Measurement was carried out in 0.62 mM KCl; 40 mM Caps (3- [cyclohexylamino] -1-propanesulfonic acid) -KOH (pH = 10); to dissipate potential use protonophore-uncoupler FCCP (p-trifortmetoksifenilgidrazon) and monensin antiporter. Swelling of mitochondria were recorded on a spectrophotometer at optical density decrease in mitochondrial suspension at 540 nm

Total activity of CK and BB-CK was determined in serum on automated biochemical Prestige 24i, using Cormay firms set.

The brains of experimental animals on day placed in Bouin retainer after wiring standard histological tissue embedded in paraffin. To examine the morphology of neurons on a rotary microtome sections were prepared in sensormotory 5 microns thick crust. Sections were deparaffinized and stained to determine the nucleic acid gallocyanin-chrome alum on Einarson. The morphometric study was carried out on Axioskop microscope (Ziess, Germany),

an increase in  $\times 40$ . Images of neurons in the sensorimotor cortex, obtained on the microscope, with the help of highly sensitive video cameras Cohu-4922 (COCHU Inc., USA) was introduced in the computer hardware and software system for digital image analysis VIDAS. Image analysis was carried out in semi-automatic mode.

Degenerating neurons were considered showing signs of karyopyknosis or cytolysis. Software density measured location and degenerating neurons surviving, the ratio of intact neurons to perishing (neurodegeneration index) and ratio of the density of surviving neurons using the medication to intact neuronal density in control group (survival index improvement). As part of the dead neurons by the time of histological studies have been phagocytosed cells, microglia, separately evaluated the relative activity of microglia index equal to the quotient of the difference in the density of surviving neurons in the difference in the density of degenerating neurons (the difference between the control group and pharmacological agents). The value of the index less neurodegeneration unit testified the predominance of the number of dying neurons surviving on the index to improve the survival and activity of microglia more units showed a positive pharmacological effect of the drug, less than one - on the negative. On the functional state of the surviving neurons was evaluated on the basis of changes in the area of nuclei and nucleoli of neuronal content of nucleic acids, nuclear-cytoplasmic ratio and the number of multinuclear cells [6].

## Statistics

Statistical analysis of the results obtained was carried out in SPSS system for Windows, Version 20 (SPSS Inc., Chicago, IL, USA). The data were presented as mean (M) and standard error ( $\pm$ SE). To compare the main parameters of groups, two-tailed Student's t-test U-test were used. To compare categorical variables between groups, Chi-square test and Fisher exact test were used. A calculated difference of  $p < 0.05$  was considered significant.

## Results and Its Discussion

Formation of experimental equivalent of multiple sclerosis - experimental allergic encephalomyelitis (EAE) [15] led to permanent disturbance of energy metabolism of brain tissue - activating anaerobic glycolysis (increased lactate / pyruvate ratio), inhibition of oxidation in the Krebs cycle (reduction of malate by 51% and isocitrate 45 %) and power shortage (a decrease of 42% of ATP, ADP, 43% AMP against increase of 82%) (Table 1). Modeling and EAE led to inhibition of mitochondria-cytosolic compensatory shunts energy production, in particular malate aspartate shunt. Malate-aspartate shuttle transports the recovered equivalents generated in the cytoplasm during glycolysis in mitochondria in ischemia. Formed in the cytoplasm under conditions of low oxygen, NADH is used to convert + oxaloacetic acid malate, and the malate penetrates into the mitochondria and is involved in the export of  $\alpha$ -ketoglutarate. This mitochondrial

malate converted to oxaloacetic acid to form the NADH available for electronic transport chain (2 protons of 3 molecules of ATP are formed). Formed from malate oxaloacetic acid is converted  $\alpha$ -ketoglutarate and aspartate.  $\alpha$ -ketoglutarate is from mitochondria in exchange for malate and aspartate to glutamate exchanged. The transfer is due to the gradient of glutamate and high intramitochondrial relationship glutamate / aspartate. The ratio of NADH / NAD<sup>+</sup> and malate / acetic acid regulated malate dehydrogenase (MDH). In modeling the EAE inhibition was observed malate-aspartate shunt, resulting in lower levels of malate by 51%, and aspartate at 40% in comparison with the group Intact. These changes appear to be secondary consequences are mitochondrial dysfunction. Confirmation of this hypothesis, studies were functional activity of mitochondria isolated from the brain neurons. Thus, in untreated animals with EAE, an increase rate of mitochondrial pore opening of 9.1 times and fall of the inner mitochondrial membrane potential by 78%.

The introduction of the experimental rats with EAE methylprednisolone did not have a significant effect on the studied parameters of energy metabolism and mitochondrial dysfunction (tables 1-2). Co-administration of methylprednisolone and citicoline exerted significant influence on some parameters of mitochondrial dysfunction and brain energy metabolism. Thus, the introduction of this combination resulted in a significant reduction of mitochondrial pore opening speed by 66% and increase the charge of the inner membrane of mitochondria by 69%.

As a result, recovery of functional activity of mitochondria brain of animals with EAE by the combination of methylprednisolone and citicoline occur significant increase in ATP levels by 15% and ADP 16% and the reduction of lactate at 27% and increased pyruvate and isocitrate 35% and 81%. The contents of malate and aspartate in the group receiving methylprednisolone and citicoline were not significantly changed.

Formation EAE causes damage to the brain neurons as evidenced by the increase in the total CK activity by 152% and BB-CK is 72% in blood serum of experimental animals. Introduction of animals with EAE methylprednisolone did not affect the performance of activity of CK and BB-CK. Introduction of methylprednisolone, together with citicoline decreased the total CPK at 38% and BB-CK is 27%, which indicates a decrease in neurodegenerative processes.

Equivalent experimental on multiple sclerosis leads to neuronal damage of sensorimotor cortex of experimental animals. Thus, in a group of untreated animals with EAE neurocyte observed density decrease is 19%, indicating that the cell death, increasing their area by 10%, about testified edema. It has also been found to decrease transcriptional processes in neurons of the sensorimotor cortex in the simulation of EAE, as evidenced by RNA decline by 21%.

Simulation of EAE led to the activation of apoptosis nero. So, in the sensorimotor area of the cortex of animals with EAE observed increase in the density of apoptotic cells and destructive at 150% (Tables 3-4). Percentage of apoptotic cells in the brain structure in animals with EAE increased from 3.4 to 15%, 7%, i.e. nearly 5 times. Administration to animals with EAE methylpredni-

solone resulted in a significant increase in the density of cortical neurons sensoromotor 3% and reducing their size by 8%, indicating a direct neuroprotective effect of hormone therapy. However, the appointment of methylprednisolone alone had no effect on the functional characteristics of neurons (RNA levels did not change) and had no effect on neuroapoptosis indicators. Administration to animals with EAE combination therapy of methylprednisolone and citicoline neuroprotection increased efficiency [12, 13, 14]. In animals with EAE treated with a combination of methylprednisolone and citicoline neuronal density increased by 9.4%, their area has reached values of intact animals, RNA analysis of increased by 8.7%. The combination of methylprednisolone and citicoline slowed neuroapoptosis of neurons of sensoromotor cortex in conditions of EAE. Thus, the density of apoptotic cells and destructive decreased by 19.5%, while the proportion of apoptotic cells decreased from 15.7% in the control of up to 9% in the group receiving methylprednisolone with citicoline.

Citicoline neuroprotective (tserakson) does not show a direct energy tropic action. In the drug has a pronounced mitoprotective effect. As shown Belenichev et al. [2] Citicoline can maintain the integrity of the inner membrane of the mitochondria, as evidenced by the recovery of its capacity. This mechanism is associated with reduction of level of cardiolipin in the inner mitochondrial membrane. Also found that citicoline, indirectly, through increasing the activity of glutathione-related ferments (glutatinreduktaza and glutatiotferaza) regulates the level of reduced glutathione. Reduced glutathione, especially mitochondria, inhibits oxidative degradation Red-Oxi - sensitive areas of the mitochondrial membrane and the formation of persistent mitochondrial dysfunction [3]. Also in the works of Belenichev it is shown that by increasing the level of reduced glutathione, can reduce reaction tserakson nitrosating stress and inhibit NO-dependent mechanisms of neuroapoptosis. Save recovered equivalents glutathione helps limit the cytotoxic effects of NO and nitrotyrosine prevent accumulation. With the level of NO associated balance of pro- and anti-apoptotic mechanisms in nitrosating stress. In conditions of excess ROS (primarily peroxyinitrite and a hydroxyl radical) oxidative modification are subjected to anti-apoptotic proteins (bcl-2 and others), and the excess of NO-radical amid increased activity iNOS enhances the synthesis of pro-apoptotic proteins (FAS and APO-1) neurodegenerative pathologies. When neurodegeneration, including EAE enhanced expression of proinflammatory cytokines (IL-1, TNF- $\alpha$ , HIF-1) and the factors responsible for the transcription NF- $\kappa$ B, AP-1, JNK, which indirectly, in particular through activation of iNOS, further enhancing the formation of cytotoxic derivatives NO, leading to increased molecular reactions of mitochondrial dysfunction and neuroapoptosis. Strengthening the neuroprotective effect of co-administration of methylprednisolone with tserekson can be explained through the prism of NO-dependent mechanisms of neuroapoptosis and mitochondrial dysfunction [1, 18].

Thus, on the basis of the foregoing, it can be concluded that the combination of citicoline and methylprednisolone limiting unproductive activity increases anaerobic

glycolysis and aerobic ATP synthesis reaction by activation of oxidation in the Krebs cycle at tricarboxylic portion (isocitrate increase). Thus the combination of citicoline and methylprednisolone not affect the activity of malate aspartatic shunt EAE conditions. Apparently, the effects of citicoline and methylprednisolone EAE focus on joint suppression of iNOS expression and activity. There are known works, that describes the effects of methylprednisolone of oppression of iNOS activity.

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**Table 1**  
**Dysfunction indicators of brain mitochondria in experimental allergic encephalomyelitis**

<i>Group of animals</i>	<i>opened mitochondrial pores, <math>\Delta E</math> (540nm)</i>	<i>mitochondrial membrane potential (Safranin-O)</i>
<i>Intact</i>	0,019 ± 0,001	50,9 ± 2,05
<i>Experimental allergic encephalomyelitis (EAE)</i>	0,193 ± 0,013 (+915%)	10,9 ± 1,21 (-78%)
<i>Experimental allergic encephalomyelitis + methylprednisolone</i>	0,186 ± 0,015	13,0 ± 1,21
<i>Experimental allergic encephalomyelitis + methylprednisolone + citicoline</i>	0,065 ± 0,005* (-66%)	18,5 ± 1,8* (+69%)

**Table 2**  
**Adenile nucleotides in the brain of animals with experimental allergic encephalomyelitis**

<i>Group of animals</i>	<i>ATP, umol/g of tissue</i>	<i>ADP, umol/g of tissue</i>	<i>AMP, umol/g of tissue</i>
<i>Intact</i>	2,80 ± 0,15	0,27 ± 0,013	0,114 ± 0,008
<i>Experimental allergic encephalomyelitis</i>	1,60 ± 0,08 (-42%)	0,153 ± 0,010 (-43%)	0,208 ± 0,021 (+82%)
<i>Experimental allergic encephalomyelitis + methylprednisolone</i>	1,67 ± 0,13	0,148 ± 0,025	0,202 ± 0,016
<i>Experimental allergic encephalomyelitis + methylprednisolone + citicoline</i>	1,81 ± 0,11* (+13%)	0,178 ± 0,011* (+16%)	0,166 ± 0,011 (-20%)

**Table 3**  
**Morphological and functional indicators of neurons of the sensorimotor cortex brain of animals with experimental allergic encephalomyelitis**

<i>Group of animals</i>	<i>The density of neurons (neuron /mm<sup>2</sup>)</i>	<i>Area neurons (um<sup>2</sup>)</i>	<i>RNA content (E<sub>on</sub>)</i>
<i>Intact</i>	1250,2 ± 25,5	83,0 ± 3,86	9,52 ± 0,33
<i>Experimental allergic encephalomyelitis</i>	1006,7 ± 10,7 (-19%)	91,5 ± 3,93 (+10%)	7,45 ± 0,62 (-21%)
<i>Experimental allergic encephalomyelitis + methylprednisolone</i>	1037,4 ± 6,8* (+3%)	84,2 ± 2,73* (-8%)	7,33 ± 0,44 (-2%)
<i>Experimental allergic encephalomyelitis + methylprednisolone + citicoline</i>	1101,4 ± 7,4* (+9,4%)	84,2 ± 2,11* (-8%)	8,10 ± 0,42 +8,7%

**Table 4**  
**Indicators of apoptosis of neurons of the sensorimotor cortex brain of animals with experimental allergic encephalomyelitis**

<i>Group of animals</i>	<i>The density of optical and destructive cells for 1 mm<sup>2</sup></i>	<i>The proportion of apoptotic cells, %</i>
<i>Intact</i>	59,4 ± 6,89	3,4 ± 0,96
<i>Experimental allergic encephalomyelitis</i>	148,0 ± 16,4 (+149%)	15,7 ± 1,7 (+361%)
<i>Experimental allergic encephalomyelitis + methylprednisolone</i>	141,6 ± 11,0 (-4%)	15,0 ± 1,0 (-4%)
<i>Experimental allergic encephalomyelitis + methylprednisolone + citicoline</i>	119,2 ± 9,68* (-19,5%)	9,0 ± 1,0* (-42%)

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