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# Molecularly-biochemical and Neuropsychophysiological Aspects of Neuroprotective Activity of Mexidol during Chronic Immobilizational Stress

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**Abstract:** It is found out that in models of rats' chronic stress an oral administration of Mexidol (100 mg/kg) during 10 days decreases a degree of DNA oxidative modification markers (8-OHG), proteins APH and KPH, hyperproduction of NO, and activity of NO-synthase. Also it changes an expression of early gene c-Fos and increases a concentration of Bcl-2 in neurons of paraventricular nuclei of the hypothalamus.

## INTRODUCTION

The decrease in longevity and quality of life directly depends on the increase in frequency of central nervous system (CNS) diseases' appearance, in an ethiogenesis of which a stress takes place. According to this, a development of techniques of pharmacological correction of emotional stress is one of essential problems of modern medicine. It is known that the one of the emotional stress pathogenesis links is an overproduction of reactive oxygen species (ROS) by bioenergetics and neurochemical systems of the brain. [3, 7, 10, 14, 18] A surplus of ROS in antioxidant deficiency conditions leads to an oxidative modification of lipids, proteins and nucleonic acids. Oxidative modification of proteinaceous components of receptors, ion channels, neuron synaptic structures leads to disorders in generation and conductance of nervous impulse, breaks synaptic transmission and, as a result, leads to an impairment of brain cognitive functions. [2, 5, 7, 9, 10, 12, 14, 15] It is also known that under the action of ROS, an activation of an expression of redox-sensitive genes takes place in a cell. Some of these genes are essential for the cell defense against toxical effects of oxidative stress, while the others, in the surplus of ROS, initiate an apoptosis. [22, 24] According to mentioned above, the use of antioxidants, nootropics and their combinations is a promising direction in pharmacotherapy of a stress and its consequences. [13] Mexidol (Ethylmethylhydroxypyridine succinate), an antioxidant with neuroprotective activity, is the dramatic example.

The medication has pronounced antioxidant, anti-ischemic, nootropic and cerebroprotective activity. [2, 4, 5, 8, 17, 18] The aim of current research is to study the neuroprotective activity of Mexidol during chronic immobilizational stress, an influence of the drug on oxidative stress, immediate early genes' expression and development of apoptosis in a brain.

## MATERIALS AND METHODS

Research has been carried out on sufficient number of experimental animals, and all manipulations have been implemented according to the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (Strasbourg, 1986 with supplements of 1998) and 'General Ethic Principles of Animal Experiments' (Kyiv, 2001). Protocols of experimental studies have been approved by the resolution of Bioethics Commission of ZSMU (protocol № 26, the 17th of December, 2010).

Experiments were carried out on 48 male rats of Vistar line with 180-190 g in weight and 6 months in age. Chronic immobilizational stress (CIS) was modeled by 2-hour long rough immobilization on a back during 10 days. [23] Mexidol (CJSC 'ALSI Pharma') was administered orally in the dosage 100 mg/kg by a metallic 0.5 ml tube during 10 days, each day 30 minutes before immobilization. On the first day of experiment, 30 minutes before stress, the conditioned reflex of passive avoidance (CRPA) had been generated in animals in a two-chamber cage. After 10 days and after 2 hours after the last stress the 'open field' test on rats has been carried out. Number of horizontal, vertical movements and looks into the 'hole' were determined. At the same time the stability of CRPA was checked by the latency period of entering the dark chamber of the cage. [15, 23] Than animals were euthanized with Nembutal narcosis (40 mg/kg). Their brainpans were being opened and brains were quickly plucked out. For biochemical study brains were washed in cooled isotonic solution NaCl (+5°C) and then were homogenized in Potter homogenizer (1500 rotations/min) during 3 minutes in 5 volumes of buffer, containing 50 mM of tris-HCl, 2 mM of EDTA and 2 mM of dithiothreitol (pH 7.4). Aliquots of these homogenates were centrifuged on Sigma 3-30 k refrigerator centrifuge (Germany) with 1000 g during 10 minutes, and then with 14000 g and temperature +4°C during 10 minutes. [11, 16, 22]

The products of protein oxidative modifications by the level of aldehyde phenylhydrazone (APH) and ketone phenylhydrazone (KPH) in the reaction of oxidized amino acid residues with 2,4-dinitrophenylhydrazone were measured in the supernatant (5 mg/ml). [12] Also, stable NO metabolites (by the amount of nitrates in Griss reaction, [16] and an activity of NO-synthase (NOS) were measured. The NOS activity was calculated according to the difference between the speeds of NADPH oxidation registered by a fluorimeter in two parallel samples – with and without N-nitro-L-arginine (the NOS inhibitor). [19] Protein concentration was measured with Bradford protein assay.

The level of oxidative DNA modification was calculated by

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**Table 1: An Influence of Mexidol on Orientative and Investigative Activity and Memory of Animals with CIS**

| Groups of Animals | Number of Horizontal Movements (in 3 min) | Number of Vertical Movements (in 3 min) | Number of Looks into the 'Hole' (in 3 min) | Latency Period of CRPA, s |
|---|---|---|---|---|
| Intact | 56,5±2,76 | 17,2±0,86 | 7.8±0,66 | 180,5±11,0 |
| CIS (control) | 10,6±2,87 | 4,3±0,27 | 2,2±0,14 | 52,4±5,6 |
| Mexidol 100 mg/kg + CIS | 22,5±2,4* | 13,2±0,54* | 3,46±0,14 | 78,8±6,4* |

* $p < 0.05$ in comparison with control

**Table 2: An Influence of Mexidol on the Amount of Products of Protein Oxidative Modifications, Stable NO Metabolites, NO-Synthase Activity in Rat Brain and Daily Expression of 8-OHG during CIS**

| Groups of Animals | APH, units/g of Protein | KPH, units/g of Protein | $NO_2^-$, μm/g of Protein | NO-Synthase, nmole/mg/min | 8- OHG, nmole/day |
|---|---|---|---|---|---|
| Intact | 0,72±0,033 | 0,58±0,011 | 5,67±0,23 | 8,44±0,33 | 97,8±12,6 |
| CIS (control) | 2,77±0,112 | 3,02±0,155 | 21,43±1,44 | 22,57±2,56 | 412,3±16,8 |
| Mexidol + CIS | 1,76±0,105* | 2,37±0,176* | 12,55±1,11* | 16,43±1,52* | 337,8±17,8* |

* $p < 0,05$ in comparison with control

**Table 3: An Influence of Mexidol on the Expression of Immediate Early Genes (C-Fos) by the Number of Fos-Positive Neurons and on the Amount of Bcl-2 in Neurons of Paraventricular Nucleus of the Hypothalamus of Animals with CIS**

| Groups of Animals | Number of Fos-positive Neurons in 1 $mm^2$ | Number of Bcl-2-positive Neurons in 1 $mm^2$ |
|---|---|---|
| Intact | 40,4±2,5 | 167,8±11,7 |
| CIS (control) | 125,44±10,7 | 83,2±5,8 |
| Mexidol + CIS | 73,54±7,4* | 114,8±7,2* |

* $p < 0,05$ in comparison with control

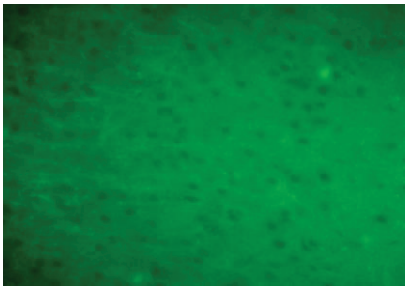

**Figure 1:** The fluorescence of Fos-positive neurons in paraventricular nucleus of the hypothalamus of intact animal group. Lens magnification x 100

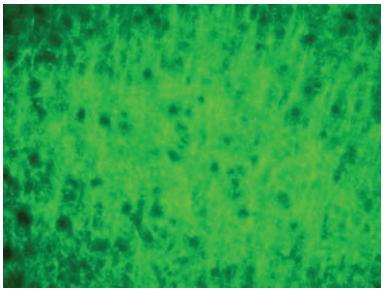

**Figure 2:** The fluorescence of Fos-positive neurons in paraventricular nucleus of the hypothalamus of control animal group. Lens magnification x 100

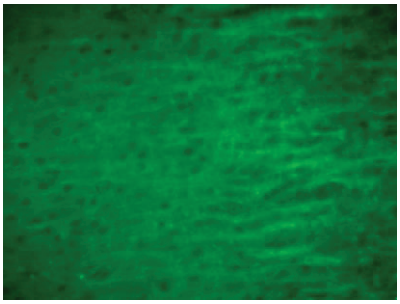

**Figure 3:** The fluorescence of Fos-positive neurons in paraventricular nucleus of the hypothalamus of animal group exposed to CIS and during Mexidol therapy. Lens magnification x 100

the daily expression of 8-hydroxyguanine (8-OHG) in a urine with chromatography method. [20]

For histochemical study a brain was fixed for 24 hours in Carnoy's fluid and then according to the standard scheme it was embedded into blocks with Paraplast-X100. After that serial frontal 14 μm histological sections of hypothalamus on rotational microtome were prepared. An immunohistochemical method of indirect immunofluorescence was used to measure c-Fos and Bcl-2 expression in paraventricular nucleus of the hypothalamus. Histological sections were taken out from Paraplast, rehydrated, three times washed for 5 minutes in phosphate buffer (pH 7.4), and incubated for 30 minutes in 2 N hydrochloric acid (T 37°C). Then they were twice washed for 5 minutes in phosphate buffer (pH 7.4), twice washed for 5 minutes in Holmes borate buffer (pH 8.4), and again four times for 5 minutes in phosphate buffer (pH 7.4). After that the material was incubated for 30 minutes in 0.1% trypsin solution in phosphate buffer (T 37°C). After incubation sections were four times washed for 5 minutes in phosphate buffer (pH 7.4) and incubated for 24 hours in dampening chamber (T 4-6°C) with primary polyclone rabbit IgG to proteins c-Fos and Bcl-2 (Sigma Chemical, USA). After incubation sections were four times washed for 5 minutes in phosphate buffer (pH 7.4) and incubated for 1 hour (T 37°C) with secondary goat antibodies to mice IgG fragment, conjugated with fluorescent dye (FITC) of Sigma-Aldrich (cat. No. F 2266). Fos-positive and Bcl-2-positive neurons were studied with the fluorescent microscope Axioskop (Ziess, Germany), the video camera COHU – 4922 (USA), and the image digital analysis computing system VIDAS–386 (Kontron Elektronic, Germany).

The choice of statistical procedures and the analysis of the mode of values distribution. The analysis of a normalcy of distribution was measured by Kolmogorov–Smirnov (D), Lilliefors, and, predominantly, Shapiro-Wilk (W) criteria. Also, as fitting criteria, the values of asymmetry and excess of data distribution were measured. When the null hypothesis that there were statistically significant differences between observed and normal distribution couldn't be rejected, nonparametric methods were used. Otherwise parametric methods were used.

**Quantitative Data Censoring**

When there were variants in studied totality that extremely differed from the entire mass of inquiries (measured according to the properties of standard normal distribution), they were excluded from further analysis. Such thing happened when these variants were higher or lower in absolute units than critical value, measured as a sum of mean value and tripled expectation sample value.

**Representation of Data**

Data was represented as a mean with a mean-square error of representatively of a sample mean.

**An Evaluation of Differences of Unrelated Sat Samples**

In cases of a normalcy of distribution of a variable, when there were more than two groups compared, in order to verify the statistical hypothesis that studied groups belonged to different general totalities, the procedure of single-factor analysis of variance was used, during which the null hypothesis that there were no statistically significant differences in set sample when $p<0.05$ was rejected, and calculated value of F-criterion was compared with a critical one for the further pair-wise comparison of groups with the use of Games-Howell criterion.

In the case of distribution that differed from normal or in the case of ordinal variable analysis the Mann-Whitney U-criterion was used for 2 unrelated sat samples, and Kruskal-Wallis H-criterion with further Games-Howell comparison – for greater number of unrelated sat samples. When the number of groups was 2, statistical significance was measured with the means of heteroscedastic Gosset U. t-criterion for unrelated groups with Bonferroni correction.

**An Evaluation of Differences of Sat Samples in Parallel Groups**

During the analysis of treatment influence on studied values in the case of normal distribution of variables the procedure of single-factor analysis of variance of repeated changes was used with further use of Newman-Keuls or Games-Howell, taking into account the plurality of comparisons.

In the cases when the distribution of studied variables wasn't normal, nonparametric analogue of analysis of variance of repeated changes was used – the Fridman criterion. In the case when there were only two groups, a comparison was made using Wilcoxon criterion.

**Chi-squared Test**

The comparison of groups by qualitative attribute and the study of a frequency of occurrence of values were carried out with the use of chi-squared criterion and an analysis of contingency Tables.

**Multifactorial Analysis of Variance of Repeated Changes and Co-variance Analysis**

In analysis of an influence of different factors on studied parameters during therapy and the analysis of dynamics of indexes in dependence on initial values, the multifactorial analysis of variance of repeated changes and covariance analysis were used.

**Correlation Analysis**

An evaluation of relation rate between pairs of independent attributes, expressed on quantitative manner, was made with the use of Pearson (r) or P. Spearman (R) coefficient of rank correlation, in dependence on the mode of variables' distribution. The confidence estimation of correlation coefficients was carried out, comparing calculated coefficients with critical (according to the properties of correlation coefficients and degrees of freedom).

**Regression Analysis**

For the determination of existence and properties of dependence between numerical variables regression

analysis procedure was used with an implementation of lineal, logarithmic, power-law, exponential, and polynomial (the second and third power) models, in an effort to achieve independent (with Darbin-Wothson criterion), normal distribution of residues (with asymmetry and excess values as fitting criteria). The final choice of regression equations was implemented with general quality criterion – the weighted sum of general accuracy criterion and general adequacy criterion. The accuracy criterion was the normalized value of the average relative approximation error, while the adequacy criterion was the normalized value of the Darbin-Wothson criterion. During the analysis of regression equations the value of the multiple correlation coefficient and the $R^2$ determination coefficient were taken into account.

Results of the study were processed with licensed statistical suite «STATISTICA® for Windows 6.0» (StatSoft Inc., No. AXXR712D833214FAN5) and «SPSS 16.0», «Microsoft Excel 2003». Separate statistical procedures and algorithms were implemented in the form of intentionally created macrosses in relevant programs.

## RESULTS AND DISCUSSION

The results of studies showed that CIS was characterized by changes in motional activity that came to decrease in horizontal and vertical activity, and in inhibition of investigative activity. An administration of Mexidol significantly in comparison with control animal group ($p < 0, 05$) increased orientative and investigative activity. The CRPA repetition, made after 10 days of daily stress, showed that the maintenance of a memory trail is impaired in control animals. Almost all rats of this group quickly entered the dark 'dangerous' chamber. An administration of Mexidol decreased the CIS-caused amnesia of CRPA and significantly increased the latency period of reflex during its repetition (Table 1).

The CIS modeling during 10 days led to development of oxidative stress in a brain of experimental animals, as is evidenced by an increase in level of marker products of protein oxidative modifications. Thus, concentrations of APH and KPH in control group of animals exceeded such figures for intact group. An administration of Mexidol significantly ($p < 0.05$) decreased the concentration of protein oxidative modifications' markers. We and other authors [5, 7, 10, 21] think that protein oxidative modification leads to the decrease in protein function in electron transport chain, ATPase activity, and selectivity of transporting pores. Changes in Red/Ox potential of mitochondrial membrane can influence the function of electron chain cascade of neuron. Mentioned above changes eventually lead to an impairment of secretory, incretory, and transport functions of neuron, and, as a consequence, to the development of cognitive deficiency. [1–8, 25] It is known that due to the activity of ROS in a cell an activation of Red/Ox-sensitive genes takes place. Many of these genes are needed for a cell defense against toxicological effects of oxidative stress. Thus, in normal concentration of oxygen in an environment (normoxia) by the action of ROS an activation of generally c-Jun and ATF-2 transcriptional factors takes place, while in condition of oxidative stress – predominantly JunB and c-Fos factors. [6, 7, 8, 22, 24, 25] An activation of exactly these transcriptional factors in condition of ROS overproduction is explained by the presence of highly sensitive to ROS cysteine residues in DNA-binding domains of JunB and c-Fos. Oxidation of their SH-residues leads to the reversible inactivation of AP-1 and NF-kB. These data is confirmed by studies of other authors [2, 7, 24, 25] which showed that emotional stress intensified an expression of c-Fos in different brain structures.

To the point of view of some scientists, hyperproduction of NO and OH· in some pathologies (ischemia, stress, neurodegenerative diseases etc) plays a crucial role in molecular mechanisms of oxidative stress and represents the trigger mechanism in oxidative destruction of cell DNA. [3, 7, 24] Oxidative modification of DNA leads to the formation of 8-hydroxyguanine (8-OHG) which has profound cyto- and genomotoxic activity with the major consequences – an impairment of molecularly-biological processes in a cell (replication and transcription). It is seen from Table 2 that CIS is accompanied by significant rise in 8-OHG concentration in daily rat urine. An administration of Mexidol in animals showed protective activity in respect of nucleic acids in oxidative stress as was evidenced by a decrease of daily 8-OHG excretion $p < 0.05$.

Recently new articles appeared that convincingly proved the role of NO in a pathogenesis of neurodestructive diseases. A significant role in hyperproduction of NO belongs to inducible no-synthase that is expressed under action of transcription factors JunB, c-Fos, and AP-1. [9, 14, 22, 24]

Our researchers found out that CIS led to an activation of NO-synthase and hyperproduction of NO as was evidenced by four-fold increase of its stable metabolites in rat brain. An administration of Mexidol in experimental animals led to normalization of these indices. This effect of Mexidol, apparently, is one of mechanisms of its neuroprotective action, since the ratio of intracellular concentrations of NO and ROS dictates the effects of these compounds on processes related to neurons apoptosis regulation. In conditions favorable for an accumulation of peroxynitrite ($ONOO^-$), NO stimulates an apoptosis, that is related first of all to an activation of kinase JNK, factors p53 and Bax, release of cytochrome C from mitochondria with further activation of caspases. [6, 7, 22, 24]

Histoimmunochemical studies showed that the number of Fos-positive neurons in paraventricular nucleus of the hypothalamus of rats with CIS was significantly higher ($p < 0.01$) that of rats of intact group (Figure 2). An administration of Mexidol in a dosage of 100 mg/kg led to significant decrease ($p < 0.01$) in Fos-positive neurons in paraventricular nucleus of the hypothalamus in comparison with control animal group (Figure 2, table 3). Also a decrease in concentration of antiapoptotic protein Bcl-2 in neurons in paraventricular nucleus of the hypothalamus was observed. It is believed [22, 24] that Bcl-2 is metal-containing protein, the scavenger of ROS, an apoptosis development blocker.

An administration of Mexidol slowed down a decrease in Bcl-2 as was evidenced by the lowering of a number of

Bcl-2-positive neurons in a paraventricular nucleus of the hypothalamus in comparison with control group ($p < 0.01$).

Thus, results of carried out research read that Mexidol has significant influence on different indices of rat resistance against CIS and has pronounced stress-protective and neuroprotective activity. The mechanism of stress-protective and neuroprotective activity of Mexidol is explained generally by its antioxidative properties and related to its ability to increase an activity of glutathione-dependent enzymes and an amount of reduced glutathione, and also to decrease the grade of protein oxidative modification by the means of direct interaction with ROS. Mexidol has pronounced membrane-stabilizing activity (increases an amount of phosphatidylserine and phosphatidylinositol), normalizes an activity of many crucial membrane-dependent enzymes – adenylate cyclase and creatine phosphokinase. [3, 7] Mexidol has protective activity on protein components of neuron membranes – receptors and ion channels, improves nervous conductivity,

synaptic transmission and increases an ability of receptor complexes to bind with ligands and to increase an activity of neurotransmitters. Thus, Mexidol improves intellectual and memory activity. [3, 6]

In conditions of antioxidant deficiency that developed in CIS, Mexidol decreased the rate of oxidative modification of DNA, proteins and lessened the hyperproduction of NO (according to concentrations of its stable metabolites) and activation of NO-synthase.

An administration of Mexidol changed expression of early gene c-Fos and content of Bcl-2 in neurons in a paraventricular nucleus of the hypothalamus in rats. The area of a paraventricular hypothalamus is a source of corticotropin-releasing factor, which, as it is known, leads to higher production of ACTH in hypophysis and, thus, activates the synthesis of steroids in adrenal glands, which are the main link in emotional stress. An activation of early genes and, consequently, an increase in number of Fos-positive neurons in a paraventricular nucleus is a marker of activation of this structure. [22, 24] Moreover, as it was shown previously, c-Fos plays a significant role in an expression of NO-synthase and, as a consequence, in a hyperproduction of stable NO metabolites, which are cyto- and genomotoxic. Expression of an early c-Fos gene plays some role in an initiation of apoptosis during CIS as was evidenced by a decrease in Bcl-2 amount. An influence of Mexidol on an expression of c-Fos and amount of Bcl-2 in rat neurons during CIS can explain the mechanism of its neuroprotective activity, particularly, by an influence on such delayed mechanisms of neuronal death after stress, as apoptosis.

## CONCLUSION

1. The modeling of chronic stress in rats by way of daily 2-hour long rough immobilization on a back after 10 days of experiment led to a disorder of cognitive and memory function, an increase in formation of products of protein and nucleic acid oxidative modifications, stable NO metabolites, NO-synthase hyperenzymemia, and to an excessive expression of immediate early genes (c-fos) and a decrease in a concentration of antiapoptotic protein Bcl-2.
2. Oral administration of Mexidol (100 mg/kg) in animals during 10 days led to a decrease in levels of oxidative DNA and protein modification markers (8-OHG, APH and KPH), and to a decrease in NO hyperproduction (concentration of stable- $NO_2^-$ metabolites) and NO-synthase activity.
3. Mexidol changed an intensity of immediate early gene c-Fos expression and increased a concentration of Bcl-2 in neurons of paraventricular nucleus of the hypothalamus in rats with chronic immobilization stress.
4. Mexidol lessened an expressiveness of cognitive and memory function in rats with chronic immobilizational stress, while increasing orientative and investigative activity and conditioned reflex of passive avoidance integrity.
5. An influence of Mexidol on expression of c-Fos and concentration of Bcl-2 in neurons of rats with chronic immobilizational stress can explain the mechanism of its neuroprotective activity, particularly, by an influence on such delayed mechanisms of neuronal death after stress, as apoptosis.

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