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Original Article

Influence of mexidol on early genomic response and morphofunctional parameters of the brain cortex sensorimotor zone neurons after arteria carotis communis occlusion

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ABSTRACT

Objective: One of the most important problems of modern neuropharmacology is a development of new routes for pharmacological correction of cerebral strokes, as a reason of significant increase in morbidity, steady loss of working efficiency, and heavy mortality. Ischemic stroke is a multifactor pathological process and occurs primarily in the midst of a mismatch of blood supply level and metabolic brain tissue processes. Medications of secondary neuroprotection (antioxidants, metabotropic and nootropic drugs, and neuropeptides) take an important place in the treatment of ischemic stroke. Nowadays, the antioxidant mexidol (2-ethyl-6-methyl-3-hydroxypyridine succinate) is widely used in clinical practice as a neuroprotector. However, its influence on morphofunctional state of neurons and genome response hadn't been studied yet. The aim of the current study was to evaluate the neuroprotective activity of mexidol by its ability to influence the mode of immediate early response genes' expression and morphofunctional neuron parameters. **Materials and Methods:** Research has been carried out on sufficient number of experimental rats. For morphological and histoimmunochemical study brain tissue of experimental animals was used. We studied: Cell composition in the area of IV-V cortex layers; density, area, and RNA concentration of normal, apoptotically, and destructively changed neurons. **Results:** Modeling of ischemic brain damage led to significant change in the genome response which was manifested by an impairment of a mode of expression of immediate early response gene c-fos. Administration of mexidol (250 mg/kg) caused neuroprotective effect. **Conclusions:** Intraperitoneal administration of mexidol for 21 days after arteria carotis communis occlusion increases c-fos gene expression, thus showing neuroprotective action of the drug.

KEY WORDS: Arteria carotis communis occlusion, c-fos gene expression, mexidol, neuroprotection

INTRODUCTION

One of the most important problems of modern neuropharmacology is a development of new routes of pharmacological correction of cerebral strokes, which is due to significant increase in morbidity, steady loss of working efficiency, and heavy mortality [1,2]. Ischemic stroke is a multifactor pathological process and occurs primarily in the midst of a mismatch of blood supply level and metabolic brain tissue processes. Studies on critical blood supply level

of a brain allowed a formulation of the "ischemic penumbra" conception [3,4]. Its clinical significance states that neuron function impairment is reversible during a short period of time (3-6 h), and a restoration of blood supply and a metabolism in this area allows a local repair of normal neuron functioning. Due to this fact, in present, some researchers and clinicians raise the question of worthwhileness of combined administration of primary and secondary neuroprotection drugs, with the aim to restore blood supply and a metabolism of neurons, and also regulate processes of cell death [3,5]. The main cause of neuron death in "ischemic penumbra" zone is an activity of the glutamate cascade and an oxidative stress. In a cascade of glutamate and oxidative neurotoxicity, one of the stages that lead to neuron death, is a change in an expression of apoptosis inductive and inhibitive genes, impairment in reductive and oxidative processes due to the blockage of mitochondrial complex [6-8]. An important role is played by so-called 'early genes' from c-fos family [9-11].

Medications of secondary neuroprotection (antioxidants, metabotropic and nootropic drugs, and neuropeptides) take an important place in a treatment of ischemic stroke. Nowadays, the antioxidant mexidol (2-ethyl-6-methyl-3-hydroxypyridine succinate) is widely used in a clinical practice as a neuroprotector. However, its influence on morphofunctional state of neurons and genome response had not been studied yet.

The aim of the current study is an evaluation of neuroprotective activity of mexidol by its ability to influence the mode of immediate early response genes' expression and morphofunctional neuron parameters.

MATERIALS AND METHODS

Animals

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 the Zaporizhzhya State Medical University (ZSMU; protocol No 26, December 17, 2010).

An impairment of cerebral blood flow was modeled by inconvertible one-way occlusion of arteria carotis communis in Mongolian gerbils (*Meriones unguiculatus*), 65-70 g in weight, that according to modern literature, data were most frequently used for a modeling of brain blood flow impairment caused by weakly developed system of collateral blood flow [12].

Animals were divided into following groups:
- Intact (n = 10);
- Control (4 days and 21 days ischemia) (n = 40);
- Animals with experimental ischemia and administration of mexidol (CJSC "ALSI Pharma", Moscow, Russia) for 4 days (250 mg/kg), intra-peritoneally (n = 10);
- Animals with experimental ischemia and administration of mexidol for 21 days (250 mg/kg), orally with the aid of the metal probe and 0.5 ml of water, intragastrically (n = 20).

Animals were sacrificed under sodium thiopental narcosis (40 mg/kg), administered intraperitoneally.

Morphological and Histoimmunochemical Studies

For morphological and histoimmunochemical study, brain tissue of experimental animals was placed for fixation into the Buen solution for 24 h, and after standard histological procedure tissue was embedded into X-TRA paraplast. After that, sections of studied brain division, 5 μm in width, from the area of gyrus postcentralis (somatosensory cortex) were prepared on rotational microtome [12].

For the study of morphofunctional state of neurons of IV-V cortex layers, histological sections were colored for detection of nucleic acids by gallocyanin-chrome alum stain (Einarson's method).

Parameters measured

- Neuron's density, a density of apoptoticaly, and destructively changed neurons (number of cells on 1 mm^2 section area);
- Cell composition in the area of IV-V cortex layers (%);
- Neuron's area, an area of apoptoticaly, and destructively changed neurons (μm^2);
- RNA concentration in neurons, in apoptoticaly and destructively changed neurons (units of optical density, U_{od}, measured as a logarithm of relation between an optical density of a cell and an optical density of intercellular substance) [12].

For histological studies, the brain parts were fixed in Carnoy's fluid for 24 h and then were filled, by the normal scheme, in blocks by paraplast X-TRA. Then, these blocks were used for preparation of 14-μ histological sections of sensorimotor cortex using microtome.

For assessment of expression of early response genes c-fos and bcl-2, histological brain sections were deparaffinized, rehydrated, thrice washed with phosphate buffer for 5 min (pH 7.4), and incubated with 2N hydrochloric acid (HCl) solution for 30 min (37°C). Then sections were washed twice with phosphate buffer for 5 min (pH 7.4), twice for 5 min with a borate buffer by Holmes (pH 8.4), 4 times for 5 min with phosphate buffer (pH 7.4) and incubated with 0.1% trypsin solution in phosphate buffer for 30 min (37°C). After incubation, sections were washed four times for 5 min with phosphate buffer (pH 7.4) and then incubated for 24 h in a humid chamber (4-6°C) with primary rabbit polyclonal antibodies IgC (1:500) to the c-fos protein (F7799; Sigma-Aldrich, St. Louis, MO, USA) or analog IgC to bcl-2 protein (1:100) (SAB4300340; Sigma-Aldrich). After incubation, sections were washed four times for 5 min

with phosphate buffer (pH 7.4) and incubated for 1 h (37°C) with secondary antibody of goat to fragment of rabbit IgG, conjugated to a fluorescent dye (FITC; Sigma-Aldrich). After the final washing with phosphate buffer (pH 7.4), sections were embedded in glycerol-phosphate buffer (9:1).

Intensity of c-fos and bcl-2 expression was determined by the density of c-fos-immunopositive neurons and bcl-2-immunopositive neurons of IV-V layers of sensorimotor cortex in sections with a high-sensitive CCD camera (model COHU-4922; San Diego, CA, USA) of fluorescent microscope Axioskop (Carl Zeiss Light Microscopy, Goettingen, Germany). Obtained material was processed by computer image analysis system VIDA S-386 (Kontron Elektronik, Eching, Germany).

Choice of Statistical Procedures and Analysis of the Mode of Values' Distribution

The normality analysis of data distribution was measured by Kolmogorov-Smirnov, Lilliefors, and, predominantly, Shapiro-Wilk criteria. Furthermore, 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. Data were represented as a mean with a mean-square error of the mean.

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. This happened when these variants were higher or lower in absolute units than the critical value, measured as a sum of the mean value, and tripled expectation sample value.

Evaluation of Differences of Unrelated Samples

In cases of normally distributed data, 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. In the case of non-normal distribution or in the case of ordinal variable analysis the Mann–Whitney U test was used for 2 unrelated samples, and Kruskal–Wallis H test with further Games-Howell comparison was used for greater number of unrelated samples.

Evaluation of Differences of Samples in Parallel Groups

During the analysis of treatment influences on studied values in the case of normal distribution 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 was not normal, nonparametric analogue of analysis of variance of repeated changes (Friedman test) was used; in the case when there were only two groups, a comparison was made using the Wilcoxon test.

Chi-squared Test

The comparison of groups by qualitative attribution and the study of a frequency of occurrence of values were carried out with the use of chi-square test and analysis of contingency tables.

Multifactorial Analysis of Variance of Repeated Changes and Covariance Analysis

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

Correlation Analysis

An evaluation of the relation rate between pairs of independent attributes, expressed on quantitative manner, was made with the use of Pearson or Spearman correlation, independence on the mode of variables' distribution.

Regression Analysis

For the determination of existence and properties of dependence between numerical variables, regression analysis procedure was used with implementation of lineal, logarithmic, power-law, exponential and polynomial (the second and third power) models, in an effort to achieve independent (Durbin-Watson test), 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 Durbin-Watson test.

Results of the study were processed using the software Statistica® version 6.0 (StatSoft Inc., Tulsa, OK, USA), SPSS® version 16.0 (SPSS Inc., Chicago, IL, USA) and MS Office Excel 2003 (Microsoft).

RESULTS

Modeling of ischemic brain damage led to significant change in the genome response which was manifested by an impairment of expression of immediate early response gene c-fos. Administration of mexidol caused neuroprotective action. Details of the results are represented in Tables 1-5.

On day 4 of the experiment, a significant decrease in a number of c-fos positive cells in relation to intact animal group occurred [Table 1]. On the 21st day of the experiment (recovery period)

slight increase of c-fos expression in comparison with the 4th day of ischemia was observed; this was related to the switch of the type of cell death from necrosis to apoptosis in the recovery period [Table 1]. On the 4th day, more than a 2-fold decrease in the number of bcl-2 positive neurons was registered, with further increase in their number on the 21st day [Table 1].

Table 1: Influence of mexidol on c-fos gene expression and on bcl-2 concentration in neurons of Mongolian gerbil's sensorimotor cortex

Animal groups	c-fos positive cells per mm²	bcl-2 positive cells per mm²
Intact animals	15.7±3	93.7±4.7
Animals with arteria carotis communis occlusion, day 4	4.8±1.7	41.5±3.7
Animals with arteria carotis communis occlusion, treated with mexidol, day 4	5±1.5	47±5.7
Animals with arteria carotis communis occlusion, day 21	8.2±2.3	33.5±2.4
Animals with arteria carotis communis occlusion, treated with mexidol, day 21	10.5±3*	52.6±4.5**

*$P<0.05$ in comparison with the respective control group

Table 2: Influence of mexidol on neurons' density (cells/mm²) in Mongolian gerbil's brain cortex (layers IV-V) (mean±SEM)

Time after ischemia	Intact animals	Animals with arteria carotis communis occlusion	Carotis communis occlusion, treated with mexidol
Day 4	1141±40	925±21	950±15
Day 21	1141±40	811±14	987±12*

*$P<0.05$ in comparison with the respective control group,
SEM: Standard error of the mean

Table 3: Influence of mexidol on neurons' area (μm²) in Mongolian gerbil's brain cortex (layers IV-V) (mean±SEM)

Time after ischemia	Intact animals	Animals with arteria carotis communis occlusion	Carotis communis occlusion, treated with Mexidol
Day 4	104.8±7.07	88.1±7.8	91.9±7.9
Day 21	104.8±7.7	83.3±8.8	101.1±9.11*

*$P<0.05$ in comparison with the respective control group,
SEM: Standard error of the mean

Table 4: Influence of mexidol on RNA content (U_{od}) in neurons of rat's brain cortex (layers IV-V) (mean±SEM)

Time after ischemia	Intact animals	Animals with arteria carotis communis occlusion	Carotis communis occlusion, treated with mexidol
Day 4	14±0.75	10.21±0.57	11.78±0.67
Day 21	14±0.85	9.71±0.47	13.81±0.77*

*$P<0.05$ in comparison with the respective control group,
SEM: Standard error of the mean

Table 5: Influence of mexidol on the density of apoptotically and destructively changed neurons (cells/mm²) in Mongolian gerbil's brain cortex (layers IV-V) (mean±SEM)

Time after ischemia	Intact animals	Animals with arteria carotis communis occlusion	Carotis communis occlusion, treated with mexidol
Day 4	21±1	274±10	267±11
Day 21	21±1	261±11	217±12*

*$P<0.05$ in comparison with the respective control group,
SEM: Standard error of the mean

Administration of mexidol during the first 4 days of ischemia did not cause significant influence on c-fos gene expression in comparison with the group of untreated animals. Administration of mexidol during 21 days after arteria carotis communis occlusion more significantly increased c-fos gene expression ($P < 0.05$). Similar dynamics were found out for bcl-2 positive neurons.

Neuroprotective action of mexidol appeared also as increasing of a density of glial cells and neurons in brain cortex on the 4th and the 21st day of the experiment, and an increase in their morphofunctional and transcriptional activity (increase in DNA concentration) with simultaneous decrease in the number of destructively changed cells [Figures 1-4 and Tables 2-5].

DISCUSSION

A change in an expression of immediate early response genes (so-called "tertiary messengers": c-fos, c-jun, krox-20, zif/268 genes, *etc.*) in the nucleus may be considered as a non-specific genome reaction on every damaging influence, particularly on ischemia. It is known that proteins of fos-, jun-, and krox-gene families play an important role in a control of cell cycle, development, growth and cell differentiation, and also determine a further fate of differentiated neurons. An expression of genes leads to the synthesis of DNA-related proteins, transcription factors that in turn cause an expression of other immediate early response genes. Thus, immediate early response genes play a role in data transmission from cell to cell. It is interesting that transcription factors may be the mediators of either neuronal death or cell survival [9,13-15].

Many authors consider c-fos as an early marker of signal systems' activation in apoptosis [9,16]; c-fos protein forms dimers with other proteins and d-jim, c-jun, activating transcription factor and activator protein 1 (AP-1) complex are created as result. The mechanism of apoptosis activation by an immediate early response gene such as c-fos and its products (e.g. transcription factor AP-1), apparently, is through the synthesis of pathological

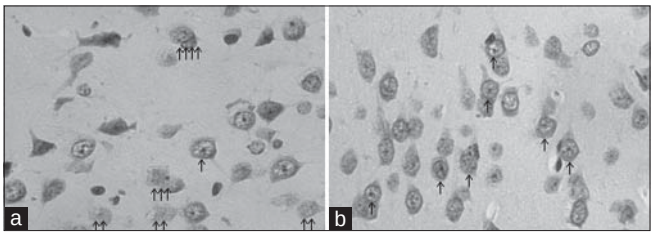

Figure 1: Neurons of sensorimotor cortex of Mongolian gerbils on the 4th day after irreversible occlusion of the common carotid artery (Lens x40). (a) neurons of IV-V layers of sensorimotor cortex of control animals: Decrease in the density of intact neurons (↑), and increase in the density of destructively modified neurons – cytolysis (↑↑), karyorrhexis (↑↑↑), and karyopyknosis (↑↑↑↑); (b) neurons of IV-V layers of sensorimotor cortex of animals after mexidol administration (250 mg/kg): Increase of density of intact neurons which contain a nucleus (↑), and decrease in the density of destructively modified neurons.

proteins or the induction of synthesis of the hypothetical apoptotic factor. Activation of immediate early response genes in a neuron may be carried out with an aid of the protein kinase cascade p21ras/MAPK or the sphingomyelinase/ceramide signal pathway. As a result, a transcription of these genes promotes development of apoptosis [17,18].

Changes in c-fos expression mode in the recovery period, determined in the current study, to our opinion, have an adaptative nature and are obligatory for the "switch" of the cell's death mode from necrosis to apoptosis. Indeed, histochemical studies of bcl-2 positive neurons showed a strong correlation between c-fos gene expression and the number of bcl-2 positive neurons. Changes in genome expression under the conditions of ischemic brain damage modeling are followed by impairment of morphofunctional state of sensorimotor cortex neurons. Thus, starting with the 4th day of ischemia, a decrease in the area and density of neurons in comparison with intacs was observed; the maximum rate was reached on the 21st day [Figures 1 and 2]. Moreover, a decrease in transcriptional activity of neurons, expressed by gradual lowering of DNA concentration in the nucleus, was detected. Such morphofunctional shifts in brain neurons led to an increase in a number of destructively changed cells [Tables 2-5].

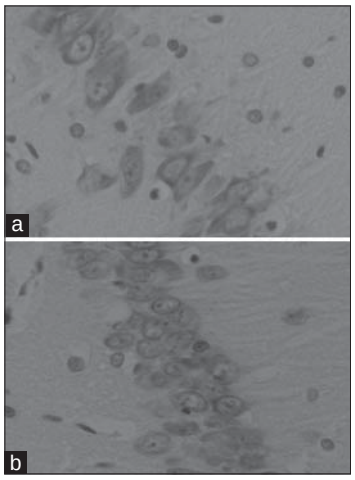

Figure 2: Neurons of sensorimotor cortex of Mongolian gerbils on the 21th day after irreversible occlusion of the common carotid artery (Lens x40). (a) Neurons of IV-V layers of sensorimotor cortex of control animals; (b) Neurons of IV-V layers of sensorimotor cortex of animals after mexidol administration (250 mg/kg).

Apparently, mexidol was able to cause an influence on molecular mechanisms of neuron death under the conditions of acute hypoxia. Such direction of activity of many neuroprotective drugs on c-fos gene expression explains the possibility of their influence on the type of cell death under conditions of brain ischemia, switching it to more "mild" apoptotic route. Such activity of neuroprotective drugs, from our point of view, is crucial, because apoptotic neuron death is an optimal, well-ordered process of destructively changed neurons' death, during which cell membranes are stabilized and cell content is utilized by formation of apoptotic bodies and their phagocytosis, without development of inflammatory response, unlike necrosis [9].

For evaluation of pathogenetic relations and for a discovery of neuroprotective action aspects of mexidol, measurements for the association of gene activity level with the number of visually changed neurons in experimental animals of control and mexidol-treated groups were carried out, with the help of scatter diagrams and binary regression analysis [Figures 5 and 6]. As a result, in experimental animals without treatment (control) association between c-fos expression level and a number of normal neurons has the form of polynomial quadratic function, showing that low number of undegenerated neurons is related with low c-fos level or dramatic increase in its activity (c-fos hyperexpression) may be the marker of programmed brain neuron death (apoptosis) under the conditions of ischemia. This U-like dependence also evidenced that normal neurons are more frequently registered in a range of 2.5-4.5 c-fos positive cells.

CONCLUSION

Intraperitoneal administration of mexidol (250 mg/kg) in rats for 21 days after arteria carotis communis occlusion increased c-fos gene expression, thus showed neuroprotective action of the drug. Mexidol administration possessed positive influence on normal functioning neurons' density, probably due to a modulation of gene activity. The efficacy of mexidol determined in the experiment is explained by its ability to influence the ischemic cascade reactions, and significantly decrease the level of post-ischemic neuron damage.

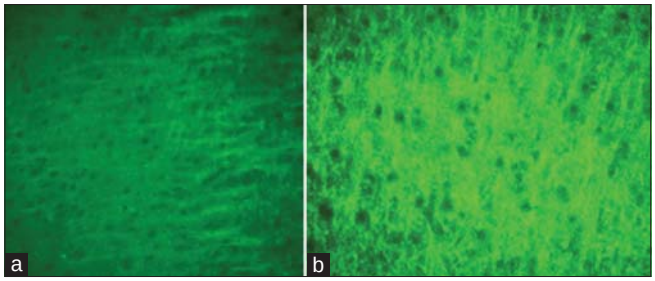

Figure 3: Expression of c-fos protein in neurons of sensorimotor cortex of Mongolian gerbils on 21th day after irreversible occlusion of the common carotid artery (Lens x20). (a) Expression of c-fos protein in neurons of IV-V layers of sensorimotor cortex of control animals; (b) expression of c-fos protein in neurons of IV-V layers of sensorimotor cortex of animals after Mexidol administration (250 mg/kg).

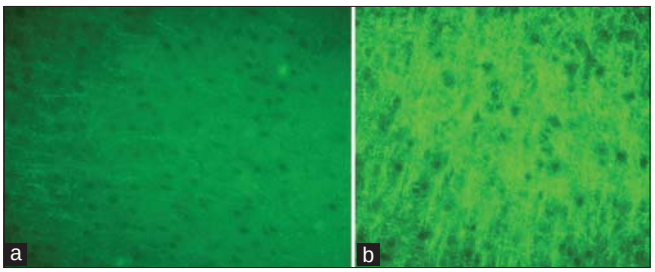

Figure 4: Expression of bcl-2 protein in neurons of sensorimotor cortex of Mongolian gerbils on 21th day after irreversible occlusion of the common carotid artery (Lens x20). (a) Expression of bcl-2 protein in neurons of IV-V layers of sensorimotor cortex of control animals; (b) expression of bcl-2 protein in neurons of IV-V layers of sensorimotor cortex of animals after Mexidol administration (250 mg/kg)

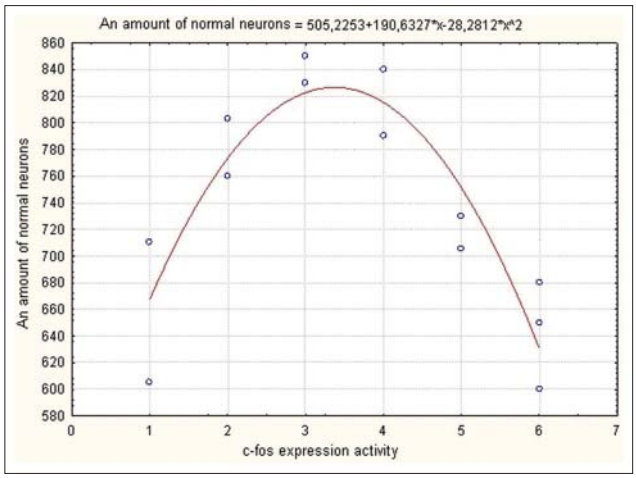

Figure 5: Regression model of the relation between c-fos expression activity and amount of structurally normal brain neurons in Mongolian gerbils of control group after arteria carotis communis occlusion

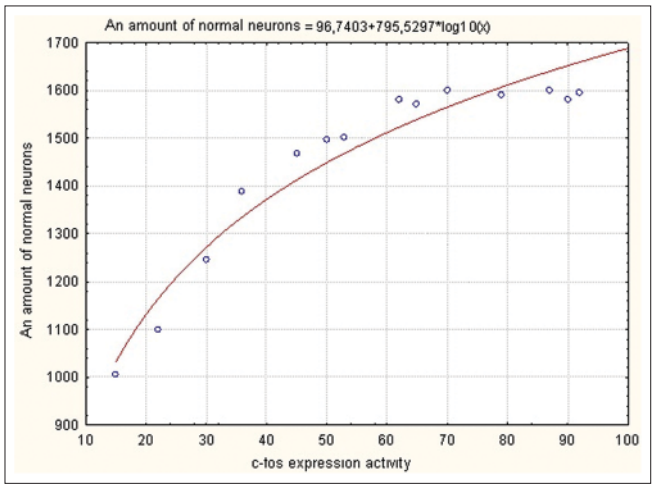

Figure 6: Regression model of relation between c-fos expression activity and amount of structurally normal brain neurons in Mongolian gerbils with arteria carotis communis occlusion after mexidol treatment

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