

# SEARCH FOR NEW APPROACHES TO TREATING PATIENTS WITH SPINAL MUSCULAR ATROPHY, 2 TYPE AS PART OF CLINICAL-EXPERIMENTAL STUDY

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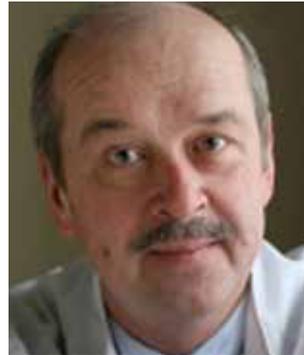
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**ABSTRACT** — in the framework of the performed clinical-experimental study it was shown that SMA-2 patients have elevated level of blood serum neurotrophins and have strong correlation between an inhibition of neurite growth in sensory ganglion neurons and neurotrophins concentration. In our opinion an influence of pharmaceuticals on tyrosine kinase receptors for the purpose of intensification of neurotrophic effect in the nervous tissue of SMA 2 patients is inexpedient.

**KEYWORDS** — spinal muscle atrophy of type 2 (SMA), blood serum, brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), organotypic culture, nerve, neurite-inhibitory effect, therapy



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## INTRODUCTION

Spinal muscular atrophy of type 2 (SMA) is autosomal recessive disorder, characterized by progressive degeneration of alfa-motor neurons of spinal cord [1]. With an incidence of 1 in 6000–10000 live births and a carrier frequency of 1 in 40–50. The disease manifests itself as a weakness of proximal muscles, pareses, respiratory insufficiency and early mortality. Spinal muscular atrophy (SMA) is an incurable disease. Thus, the development of new approaches to symptomatic treatment, which may be related to augmentation of neurotrophic regulation, is of interest at this time.

## MATERIALS AND METHODS

Clinical-neurologic and neurophysiological examination of 12 patients with 2 type SMA was carried out. Level Beta-NGF, BDNF, CNTF in 12 patients (SMA 2 type) was determined in serum using ELISA Kit (RayBiotech, Inc). The experiments were performed in the organotypic culture of a 10–11 day old chicken embryos dorsal root ganglion. Study blood serum in an organotypic tissue culture.

The research was performed on 600 explants of dorsal root ganglia of 10–12-day old chicken embryos. The culture media contained Eagle's Basal Medium with Earle's salt (BME) supplemented with 2 mg/ml D-glucose, 10 ng/ml insulin, 2 mM glutamine, 75 IU penicillin and 15% bovine fetal serum. The explants were grown on collagen at 36.5° C. Growth of the neurites was investigated using a phase-contrast microscope. The square index (SI) was calculated as the ratio of

the whole explant square area (peripheral growth zone included) to the central zone square area. The Student's criteria were used for statistical analysis. Data are expressed as means + SE. Differences were considered significant when  $P < 0.05$ . SI was expressed as a percentage, the SI of control explants was set as 100%. To visualize objects, microscope «Axio Observer ZI» («Carl Zeiss», Germany) and program ImageJ were used.

The work was based on the following methods of statistical analysis: determination of numerical characteristics of variables; estimation of conformity of empirical law of distribution of quantitative variables to theoretical law of Gaussian distribution according to Shapiro-Wilk test; an estimation of influence of qualitative factor on a dispersion of quantity indicator using ANOVA dispersion method, an estimation of a force and direction of linear relationship between the quantity indicators using parametrical Pearson correlation coefficient, nonlinear relationship — using Spearman's correlation coefficient. Description of quantitative signs was carried out using arithmetic mean value and standard deviation. Zero statistical hypothesis was rejected at significance value  $p < 0.05$ . The statistical analysis was carried out using STATISTICA 8.0 package (StatSoft, Inc., USA).

## RESULTS

12 patients with 2 type spinal muscular atrophy were examined, among them: 4 girls and 6 boys at the age from 8 up to 12 years old. All patients with 2 type SMA have been under medical observation for 3 years, during this period of time the disease was progressing. Motor defect was manifested since birth. Genetic defect was identified at the long arm of 5th chromosome (within the interval between D5S629 and D5S557). Clinical-neurologic picture includes flaccid pareses of hands and feet with prevalence of the process in the proximal parts, active movements were only in distal parts of the hands, neck muscles, mimic and respiratory muscles. There were generalized fibrillations and fasciculations of the muscles, intense diffuse hypomyotonia. 85% of children had intense atrophies of intercostal muscles with respiratory insufficiency and minor bulbar disorders. Changes of osteoarticular system were manifested as intense contractures of large joints of extremities and kyphoscoliosis. Functions of pelvic bodies were normal. There were no sensitivity and cognitive disorders.

The results of enzyme-linked immunosorbent assay showed that NGF (nerve growth factor) level ( $3899 \pm 1058$  pg/ml) in blood serum of patients with type 2 SMA was significantly ( $p < 0.001$ ) higher than in the control group ( $782 \pm 582$  pg/ml). The values of NGF levels in blood serum of the control group were

in the range from 110 pg/ml to 2237 pg/ml. Meanwhile, in patients with type 2 SMA they were in the range from 1387 pg/ml to 5411 pg/ml. We detected that patients with type 2 SMA had higher levels of NGF in blood serum. BDGF (brain-derived growth factor) level ( $36653 \pm 3606$  pg/ml) in blood serum of patients with type 2 SMA is significantly ( $p < 0.05$ ) higher than in the control group ( $27313 \pm 7260$  pg/ml). The analysis of parameter scatter detected that BDGF level in blood serum of the control group was ranging from 16040 pg/ml to 41960 pg/ml, while in patients with type 2 SMA — from 22523 pg/ml to 63700 pg/ml. The analysis of CNTF (ciliary neurotrophic factor) in blood serum did not reveal any statistically significant differences between control and study groups ( $23.0 \pm 14.3$  pg/ml versus  $21.3 \pm 13.2$  pg/ml, respectively). CNTF values in the control group ranged from 1.1 to 62.9 pg/ml, while in type 2 SMA patients - from 3.1 to 49.7 pg/ml. The following patterns of neurotrophin changes were detected: BDGF and NGF levels in blood serum of patients with type 2 SMA were significantly higher than in the control group; CNTF level did not significantly differ between control and study groups, but the values of this protein were within age limits, and no deficit of it was detected in the study group.

## EXPERIMENTAL STUDY

The next study comprised a series of experiments, which purpose was to analyze the impact of the blood plasma of type 2 SMA patients on the growth of axons of sensory ganglia in 10–12-day chicken embryos using the method of organotypic tissue culture. The following series of experiments was aimed at investigation of an influence of blood plasma of patients with 2 type SMA on growth of neurons of dorsal root ganglia of 10–12-day chicken embryos. After three days of culturing in the control and experimental explants of dorsal root ganglia there are two zones: central, consisting of not erratic differentiated neuroblasts and peripheral, so-called growth zone. The growth zone of explants of dorsal root ganglia contains mostly growth of neurites (processes of nervous cells), to a lesser degree — migrating and proliferating fibroblast-like and glial cells. Blood serum of 5 patients with type 2 SMA was researched in a wide range of dilution (1:100-1:2). In dilutions 1:2, 1:10, 1:50 the serum of patients blocked completely the growth of dorsal root ganglion neurites. When adding in cultural medium blood serum in dilution 1:70, a positive neurite inhibitory effect was observed. Area index of the studied explants was below the control value on the average by 25% and has made  $75.5 \pm 7.4\%$  (Fig. 1). Further dilution of blood serum practically did not affect the growth

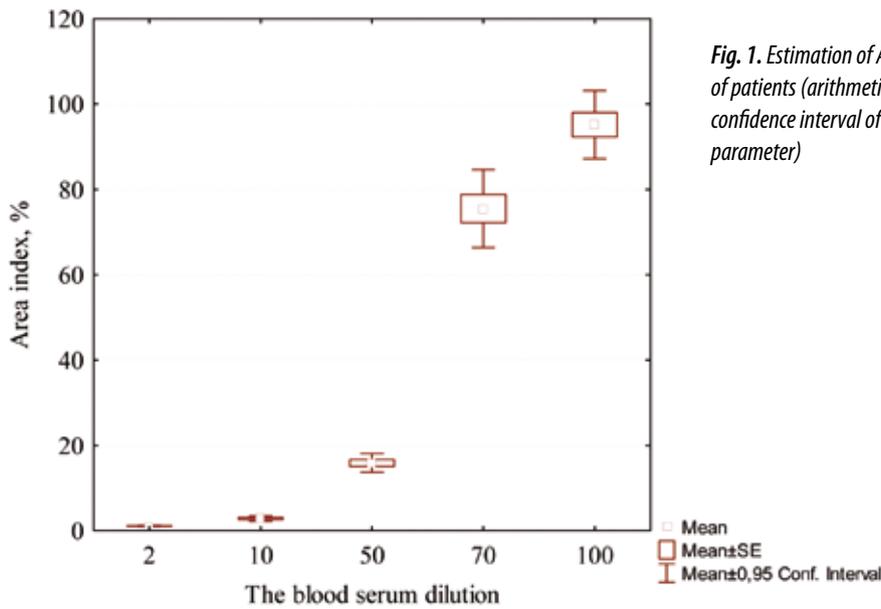


Fig. 1. Estimation of Area index in studied blood serum dilutions of patients (arithmetic mean values are shown, error bars – 95% confidence interval of mathematical expectation of Area index parameter)

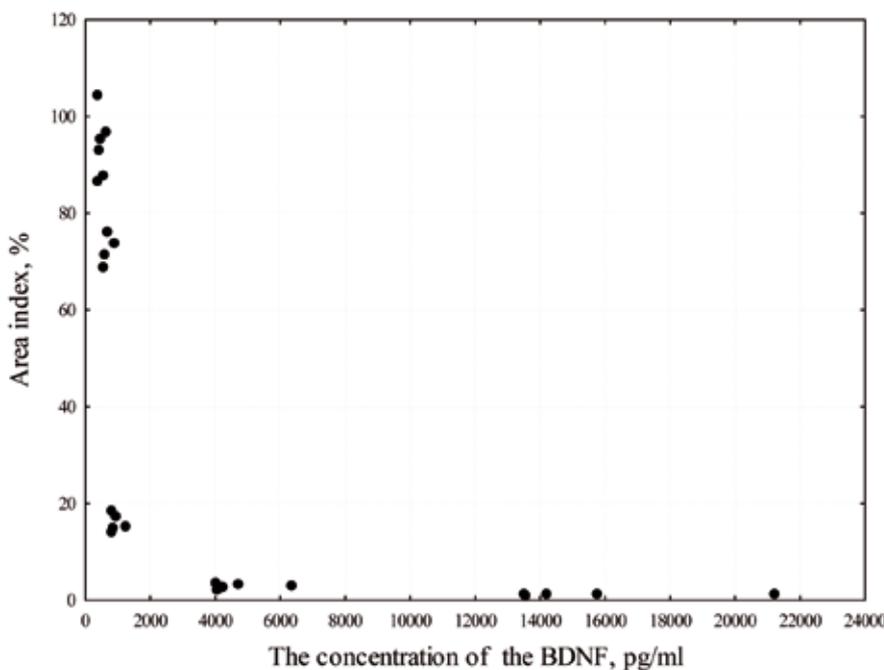
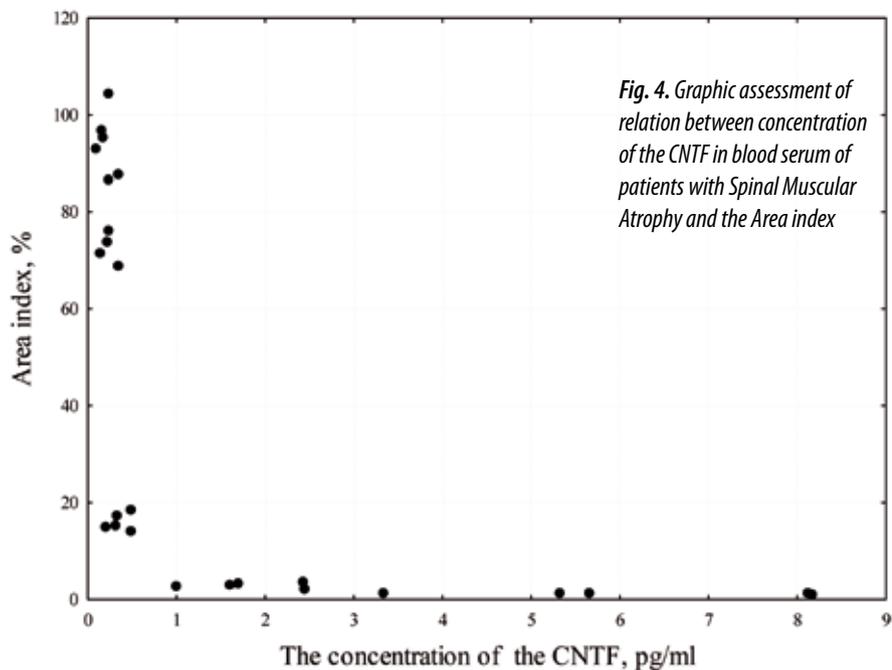
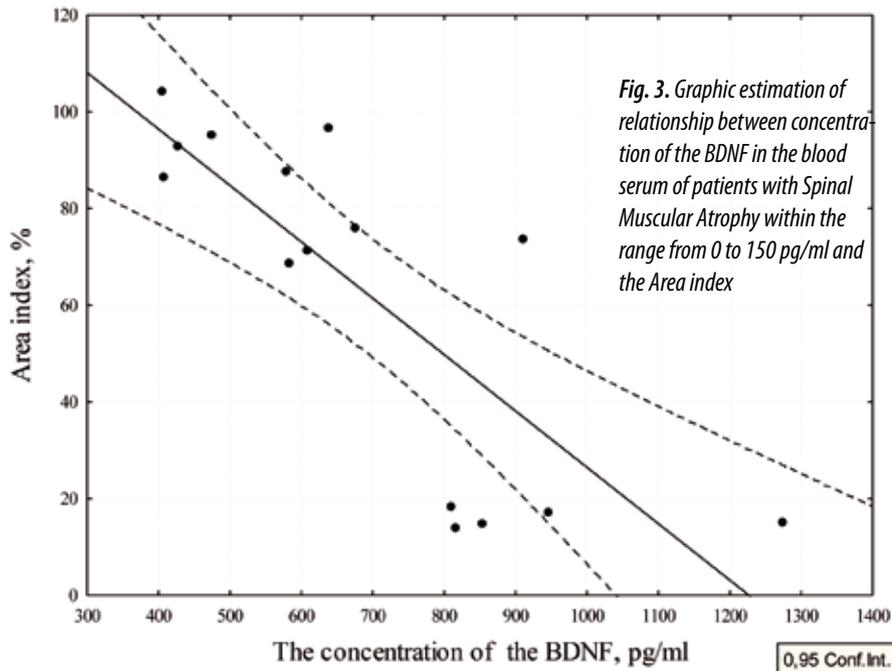


Fig. 2. Graphic assessment of relation between concentration of the BDNF in blood serum of patients with Spinal Muscular Atrophy and the Area index

of explants. The carried out studies have shown that blood serum of patients with 2 type SMA dose-dependently inhibits growth of neurites of sensory neurons of spinal ganglions. Results of estimation of the Area index for studied dilutions were analyzed using dispersion analysis. It is shown that blood serum dilution factor statistically significantly (F-test  $F=489,2$ ;  $p < 0,001$ ) influences the area index value registered in the study.

Correlation of clinical-laboratory and experimental data. Our previous study showed the presence of correlation relationship between the NGF level in

the blood serum of patients with SMA and AI (area index) [2]. Statistically significant ( $p < 0,001$ ) strong inverse (Spearman  $R = -0,90$ ) correlation relationship was detected. Thus, it was revealed that the degree of inhibition of axon growth in spinal ganglia of chicken embryos depended on the NGF level in the blood serum of patients with type 2 SMA. In this study we conducted the correlation analysis between BDGF and CNTF parameters in the blood serum of patients with type 2 SMA and AI. To evaluate the type of statistical relationship between the BDGF level in the blood serum of patients with SMA and IA, a

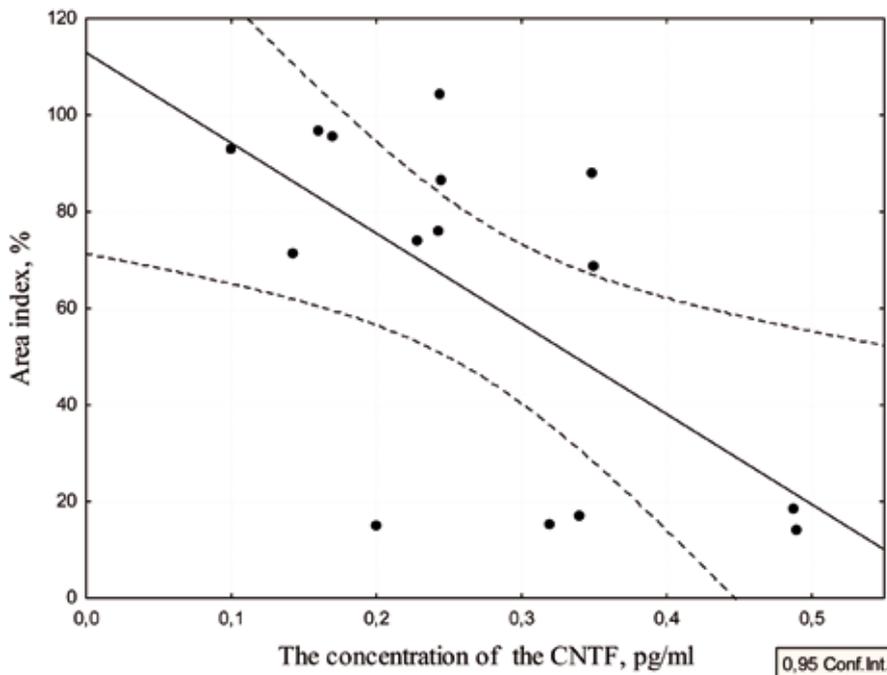


scatter diagram was plotted for the parameters described (Fig. 2). The analysis of the diagram revealed the presence of the non-linear negative correlation relationship between the parameters. The quantitative evaluation conducted using the non-parametric Spearman correlation coefficient showed the presence of the statistically significant ( $p < 0.001$ ) strong inverse (Spearman  $R = -0.94$ ) correlation relationship between the parameters. The character of the correlation field on the scatter diagram allowed to suppose the presence of partial linear connection between the BDNF level in the blood serum of patients with

SMA and AI in the level interval from 0 to 1500 pg/ml, which was confirmed when plotting the additional scatter diagram for this interval of the BDNF levels (Fig. 3). The quantitative evaluation of linear relationship showed the presence of the statistically significant ( $p < 0.001$ ) strong inverse (Pearson correlation coefficient  $r = -0.80$ ) correlation relationship between the BDNF level in the blood serum of patients with SMA and AI in the level interval from 0 to 1500 pg/ml. Likewise, the relationship between the CNTF level in the blood serum of patients with SMA and AI was analyzed. The character of the correlation field on the scatter diagram plotted (Fig. 4) revealed the presence of the non-linear negative correlation relationship between the parameters. The quantitative evaluation conducted using the non-parametric Spearman correlation coefficient showed the presence of the statistically significant ( $p < 0.001$ ) strong inverse (Spearman  $R = -0.88$ ) correlation relationship between the parameters. While the CNTF levels in the blood serum of patients with SMA exceeded 1 pg/ml, the area index approached minimal values. While the CNTF levels were below 0.5 pg/ml, the AI parameter increased. Plotted scatter diagram for the interval of CNTF levels from 0 to 0.5 pg/ml proved the hypothesis about the presence of the partial linear connection for the parameters mentioned (Fig. 5). The quantitative evaluation of linear relationship showed the presence of the statistically significant ( $p = 0.014$ ) strong inverse (Pearson correlation coefficient  $r = -0.62$ ) correlation relationship between the CNTF level in the blood serum of patients with SMA and AI in the level interval from 0 to 0.5 pg/ml. Thus, it was revealed that the degree of inhibition of axon growth in spinal ganglia of chicken embryos correlated with the BDNF and CNTF levels in the blood serum of patients with type 2 SMA.

## DISCUSSION

Our study showed that patients with type 2 SMA had increased levels of the NGF and BDNF neurotrophins,



*Fig. 5. Graphic estimation of relationship between concentration of the CNTF in the blood serum of patients with Spinal Muscular Atrophy within the range from 0 to 150 pg/ml and the Area index*

while the CNTF levels in them were normal. Presumably, the increase of neurotrophin synthesis is related to the development of compensatory & adaptive processes aimed to increase the reparative function of the nervous tissue. But, according to our studies, the increased level of neurotrophins does not lead to restoration or partial compensation of lost motor functions in patients with type 2 SMA. The experiment in the organotypic tissue culture showed that the serum of patients with type 2 SMA inhibited the axon growth in sensory ganglia [3]. The strong correlation relationship was detected between the fact of inhibition of axon growth in neurons of sensory ganglia and the NGF, BDNF, CNTF levels in the blood serum of patients with type 2 SMA. These data were confirmed by the results of electroneuromyography in patients with type 2 SMA, which showed the absence or decrease of reinnervation process [4].

## CONCLUSION

At present there are active attempts to treat neurodegenerative diseases using neurotrophins or by activating receptors, through which neurotrophins exercise their biological function [5, 6]. Probably, this therapeutic direction is important for diseases which are accompanied by the deficit of growth factors, but no deficit of neurotrophins (BDNF, CNTF, NGF) was detected in the blood serum of patients with type 2 SMA. The results of this study show that excessive stimulation of tyrosine kinase receptors using pharmacological drugs to augment the neurotrophic effect in

the nervous tissue of patients with type 2 SMA is not considered an efficient therapeutic approach.

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