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Some Aspects of in Vitro Studies of Neurotoxicity: From Choice of Biochemical and Molecular Markers to the Confirmation of Their Informativity

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Abstract

The use of laboratory animals in pharmacological and toxicological studies is axiomatic from the point of experimental science and will always be conducted. But these studies are laborious, require additional personnel, the presence of a vivarium, cause the suffering of animals and their death. Therefore, since the late 80's of the last century in the scientific community of Europe and the US actively discusses the issue of toxicological studies in vitro and, in particular, neurotoxicological studies in vitro. But the use of various molecular and biochemical markers of the central nervous system in neurotoxicological studies in vitro, as well as the morphometric parameters of a neuron, can be difficult, because they can be changed by neuroprotective, and not neurotoxic, action of a potential drug. That's why, it is necessary to identify and use molecular or bioche-

mical markers, that make it possible to clearly judge about only the neurotoxicity of the drugs.

We first discovered a correlation of the expression of 70 kDa protein-chaperones with the disruption of the functioning of the glutathione unit of the thiol-disulfide system on the background of injection of the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). Injection of MPTP caused a decrease in the reduced form of glutathione and indirectly led to the deprivation of HSP_{70} protein expression. The revealed mechanism can be one of the manifestations of the neurotoxic effect of some neuropsychoric drugs.

We revealed statistically significant linear dependence of the development of neurological deficit from the functioning of the conjugate system "nitric oxide - reduced thiols". The obtained results reveal the importance of the neuron glutathione system as an important target of neuroprotective therapy in ischemic stroke and are the experimental justification for the clinical application of thiol-disulphide system modulators. The coefficient of the ratio of nitrotyrosine to reduced glutathione can be used for the purpose of early laboratory diagnosis of cognitive-mnestic disorders in patients with cerebral pathology.

Thus, we identified specific markers for in vitro experiments, that had the greatest potential in providing information on some important mechanisms of neurotoxicity.

Keywords: neurotoxicity, in vitro studies, markers, reduced glutathione, nitrotyrosine

Introduction

Since the 90s of the last century, medicines that affect on the central nervous system (antipsychotics, antidepressants, hypnotics, etc) occupied the leading place by the frequency of appointments and turnover of sales in industrialized countries [1]. Nowadays, pharmacological ingredients that can modulate the activity of the brain and its metabolism are actively used as BAAs and food additives [1].

During the same period, the results of a lot clinical studies have been published, that convincingly demonstrated CNS adverse reactions of such drugs as β-adrenoblockers, modulators of the renin-angiotensin system, contraceptives, etc [2]. All these focuses the attention of pharmacologists and toxicologists on the problem of the neurosecurity of medicines and stimulated more in-depth studies of neurotoxicity. But carrying out of large-scale studies of the neurotoxicity of potential medicines is limited by their high cost and the problem of the ethical use of laboratory animals.

The use of laboratory animals in pharmacological and toxicological studies is axiomatic from the point of experimental science and will always be conducted. But these studies are laborious, require additional personnel, the presence of a vivarium, cause the suffering of animals and their death. Therefore, since the late 80's of the last century in the scientific community of Europe and the US actively

discusses the issue of toxicological studies in vitro and, in particular, neurotoxicological studies in vitro [3, 4].

However, the nervous system is very complex and the multifunctional and our knowledge of neurometabolism and the molecular-biochemical processes involved in the neurotoxicity of chemical compounds and potential drugs are limited. Thus, it is hard to predict how adequate the system of in vitro methods (the culture of neurons, suspension of neurons, a suspension of mitochondria, a synaptic, etc.) will reflect changes in the living organism and its nervous system. Therefore, it is difficult to develop a in vitro test studies, that could replace in vivo test systems.

In vitro studies are good for the study of pharmacological and toxicological processes in a more isolated context and are most successfully used to elucidate the mechanisms of toxicity, the identification of target cells for neurotoxicity and the identification of complex molecular biochemical changes caused by drugs [5-7]. The use of various molecular and biochemical markers of the central nervous system in neurotoxicological studies in vitro, as well as the morphometric parameters of a neuron, can be difficult, because they can be changed by neuroprotective, and not neurotoxic, action of a potential drug. That's why, it is necessary to identify and use molecular or biochemical markers, that make it possible to clearly judge about only the neurotoxicity of the drugs.

As parameters of neurotoxicity, neuroapoptosis indices proved to be quite good. Obviously, in order to study neurotoxicity in in vitro experiments, it is necessary to use markers (molecular or biochemical), which concentration significantly varies with the implementation of specific mechanisms of neurotoxicity. For example, during studying the neurotoxicity in vitro of some anthracycline-type cytostatics were found such markers for the manifestation of their neurotoxicity as bcl-2 anti-apoptotic proteins and a heat shock protein with a mass of 70 kDa. These data were first obtained by a group of researchers led by Corresponding Member. NAS and AMN of Ukraine, prof. I.Chekman during studying of the effect of doxorubicin on the brain and myocardium and correction of these disorders with a new drug with endothelioprotective and neuroprotective properties "Angiolin" [1]. During incubation of a suspension of neurons with a number of psychostimulants and nootropics in vitro, such markers as antibodies to the phencyclidine site of NMDA (NR2 test) was found [1]. The use of glutathione reduced (GSH) and nitrotyrosine as markers of neurotoxicity, nowadays, is considered. Over the past decade, fundamentally new features of the involvement of glutathione-dependent enzymes, glutaredoxin and glutathione itself in the processes of neurotoxicity and neuroprotection have been revealed [1, 2]. GSH is an important intracellular antioxidant, plays a special role in maintaining cellular redox status through participation in thiol-disulphide metabolism, that provides regulation of a number of cell functions, including regulation of gene expression, activity of individual enzymes and enzyme systems. The preservation of the optimal ratio for the nerve cell GSH / GSSG is essential for its viability. Decrease of GSH level below the norm can serve as an indicator of neurotoxicity at which there is a disturbance of the cellular redox status and changes in the redox-dependent

regulation of genes. Violation of the intracellular balance of GSH is observed in the manifestation of neurotoxic effects of antibiotics, heavy metal salts, phenylalkylamine derivatives. It is known that the reduced glutathione is a neurotransmitter and neuromodulator (in micromolar concentrations it is an agonist of glutamate receptors, in millimolar concentrations modulates the SH-groups of NMDA receptors) [8]. Therefore, the decrease in GSH and the deprivation of mRNA for GSH is an important marker of the neurotoxicity of many psychostimulants.

Exerimental Part

We first discovered a correlation of the expression of 70 kDa protein-chaperones with the disruption of the functioning of the glutathione unit of the thiol-disulfide system on the background of injection of the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). Injection of MPTP caused a decrease in the reduced form of glutathione and indirectly led to the deprivation of HSP_{70} protein expression. The revealed mechanism can be one of the manifestations of the neurotoxic effect of some neuropsychoric drugs.

Simulation in vitro of neurotoxicity by addition into the incubation neuronal suspension of MPTP caused generation of reactive oxygen species and enhanced formation of nitric oxide and its derivatives.

The provided studies showed that beginning from the early observation periods (on the 15th minute of incubation), an enhanced synthesis of NO started, stimulation of the production of which is carried out due to the hyperactivation of the neuronal isoform of NO synthase. The activity of both constitutive isoforms (neuronal and endothelial) directly depends on the intracellular concentration of calcium ions, and is significantly enhanced by the development of glutamate excitotoxicity, which caused an increased of Ca^{2+} influx into the cell.

Synthesized NO interacts with aliphatic and aromatic amines to form N-nitroamines. Thus, the nitrite content increased on the 30th minute of observation and excessed of the initial values on 2.6 times at the end of the observation. Similar data were obtained for the level of nitrotyrosine, whose concentration was 1.72 times higher than the initial values on 60th minute of observation. In parallel with the formation of nitrosative stress, there was a disturbance in the functioning of the antioxidant system, that manifested in the accumulation of free radicals, and as a consequence, in increasing of the level of products of the oxidative modification of protein molecules. The increase in the content of aldehyde and ketone derivatives of proteins was recorded on 30th minute - by 23.4 and 26.2%, respectively, relative to the values of the control suspension. Cellular enzymes are also susceptible to oxidative modifications in these conditions. We observed a decrease of the activity of SOD on 42.1% after 60 minutes of observation. This enzyme plays a key role in neutralizing the superoxide radical that is accumulating in the tissues in large quantities. At the same time, some of the synthesized NO combined with highly toxic superoxide radical and formed a peroxynitrite molecule. In this case, firstly, significant amounts

of highly neurotoxic $ONOO^-$ accumulate, and secondly, the bioavailability of NO is significantly reduced. Nitrites interact with cysteine with formation of S-nitrosocysteine and with glutathione with formation of S-nitrosoglutathione. S-nitrosoglutathione is the main transport form of NO.

Some studies have established that NO transport occurs with the formation of N_2O_3, which then nitrosylates thiols, that depleting the stores of glutathione and disturbing of the thiol-disulphide balance. During our studies we found that accumulation of oxidized glutathione was in 3.3 times higher relative to the control series. In the same time there were established decreasing of GSH on 2.9 times and inhibition of the activity of enzymes of glutathione metabolism.

Once neurotoxicity markers are determined in in vitro studies, they should be confirmed by studies on the whole organism. In our studies, it was shown that a change in the concentration of the mentioned markers of neurotoxicity well correlates with the degree of neurological disorders in animals. The established dependence of the studied parameters on the higher degree of neurological disorders, is apparently associated with the development of oxidative and nitrosating stresses and disruption of the compensatory possibilities of the organism. In addition, under the influence of ROS the HSP70 proteins themselves are oxidative modified. As a result of statistical analysis of the data, a correlation was established between the obtained indices. To determine the strength and statistical significance of the correlation, the Pearson coefficient (r) was used. It has been established that the index of neurological deficit is closely related to the level of heat shock proteins 70 both in the cerebral cortex (r = -0.914) and in the hippocampus (r = -0.813) (fig. 1 and 2).

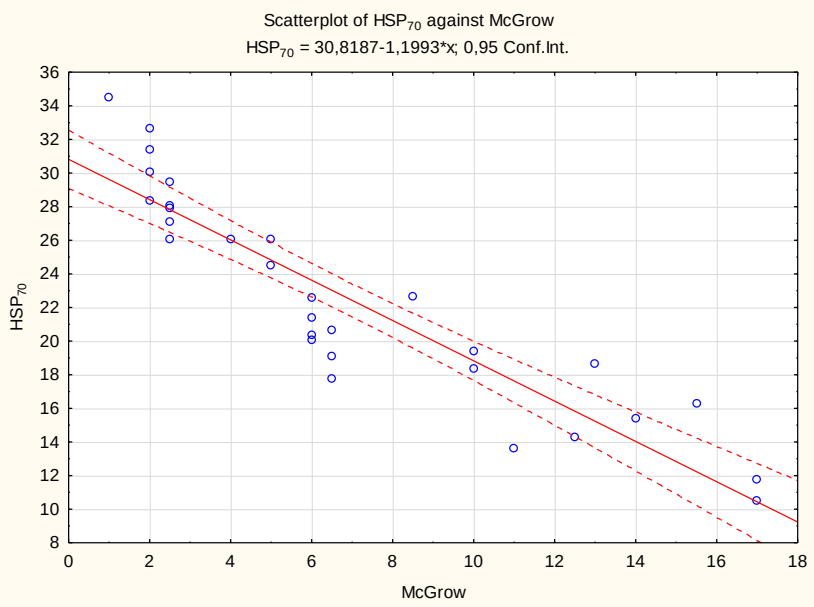

Figure. 1. Dependence of neurological deficiency index from HSP_{70} content in the brain cortex of experimental animals

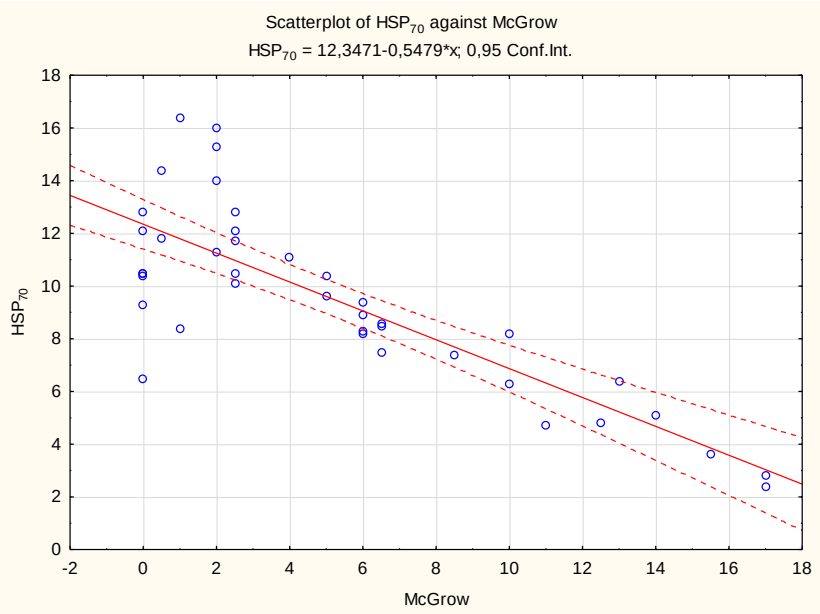

Figure. 2. Dependence of neurological deficiency index from HSP_{70} content in the hippocampus of experimental animals

Surface 3D graphics allow us to visualize a close relationship between the severity of oxidative stress (nitrotyrosine content) and the level of expression of HSP_{70}, and the formation of a neurological deficit on the 4th day of experimental ischemic neurodestruction. On the figure 3 is represented 3D graphic of such dependence in the brain cortex, on the figure 4 - in the hippocampus.

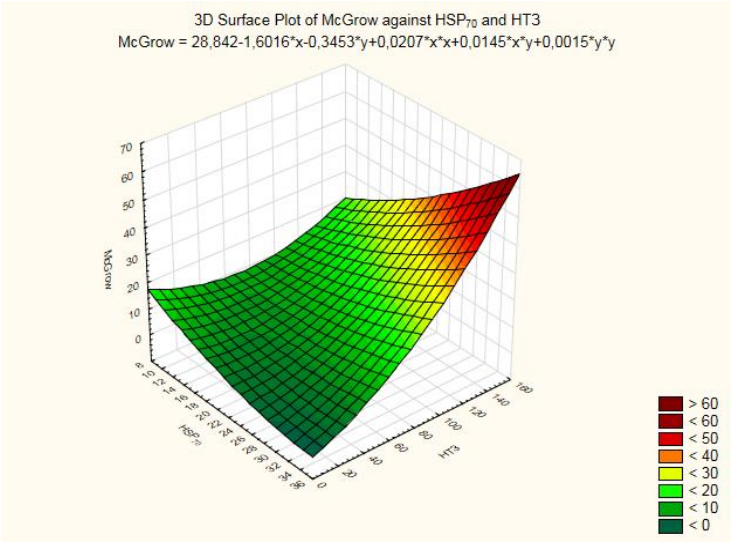

Figure. 3. Conjugation of changes of HSP_{70} and nitrotyrosine levels in the brain cortex with development of neurological deficit of experimental animals

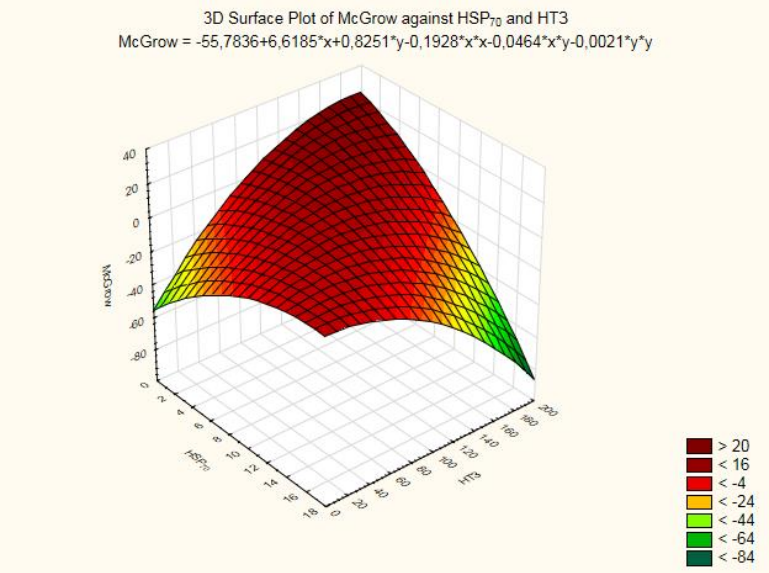

Figure 4. Conjugation of changes of HSP_{70} and nitrotyrosine levels in the hippocampus with development of neurological deficit of experimental animals

During calculating of the linear regression, we used the neurological deficit index as the dependent variable and concentrations of nitrotyrosine and HSP_{70} in the cerebral cortex and hippocampus as independent variables. Obtained results indicated about significant accuracy of the linear model. Further studies on the confirmation of selected markers (HSP70, nitrotyrosine, reduced glutathione) of assessment of the extent of damage to the CNS, confirmed the correctness of our choice and allowed to reveal the relationship between the ratio of reduced glutathione and neurologic disorders in animals with ischemic brain damage.

Simulation of ischemic neurodestruction caused the formation of neurological symptoms in all experimental animals, that was found by calculation of points of the neurological deficit by McGrow scale. Proceeding from this, all operated animals were divided into 3 groups: the first - animals with mild neurologic symptoms, the second - the middle degree, the third - the group with a severe degree of severity of violations.

Produced biochemical studies showed a clear dependence of neurological disorders from the studied parameters (Table 1).

In the cytosol fraction of brain tissue of animals with mild neurologic deficit (mean score 2.35), a decreasing of the content of GSH on 47.6% was noticed. In the same time we found accumulation of nitrotyrosine on 94.7% in comparison with intact animals. All these indicated to the development of oxidative and nitrosative stress. Further aggravation of neurological disorders was accompanied by a further progression of pathobiochemical reactions. Thus, in group 2, that was characterized by an average degree of severity in the McGrow scale, increase in the neurological score on 4.1 units occurred against a background of a 56.0% dec-

rease in the pool of reduced glutathione and an increase in nitrotyrosine on 40.1% in comparison with group 1 indices. We noticed a significant accumulation of nitrotyrosine in the brain tissues of animals with severe neurologic disorders. Nitrotyrosine is the marker of the accumulation of the most toxic product of nitrosative stress - peroxynitrite. The level of this indicator was in 3.76 times higher than in the intact group and was in 38.2 and 93.7% higher in comparison with values of groups 2 and 1, respectively.

Table. 1. Neurological deficit and biochemical parameters of experimental animals

Group of animals	McGrow Stroke-index	Glutathione reduced. (GSH), mcmol/ g of protein	Nitrotyrosine, nmol/ g of protein
Intact (healthy animals)	0	3,95 ± 0,05	5,3 ± 0,13
Group 1 (animals with mild neurological symptomatic)	2,35 ± 0,25	2,07 ± 0,05*	10,29 ± 0,65*
Group 2 (animals with middle degree of neurological symptomatic)	6,45 ± 0,22*#	0,91 ± 0,05*#	14,42 ± 0,51*#
Group 3 (animals with severe degree of neurological symptomatic)	11,8 ± 0,39*#&	0,34 ± 0,04*#&	19,93 ± 0,34*#&

Remark: * - $p<0,001$ in comparison with intact animals
- $p<0,001$ in comparison with group 1
& - $p<0,001$ in comparison with group 2

The enhanced formation of peroxynitrite was caused by the displacement of thiol-disulfide balance in neurons. The reduced thiols are able to bind the active forms of NO and prevent the formation of peroxynitrite, that limiting its toxic effect. This statement was confirmed by our research. Decreasing of the concentration of reduced glutathione proceeded in parallel with the accumulation of nitrotyrosine and the formation of a neurological deficit (table 1). In group 3 content of reduced glutathione was in 11.6 times lower than in intact animals, that ultimately led to the development of a persistent neurologic deficit (11.8 ± 0.39 points).

We investigated the character of the distribution of the studied variables for the statistical evaluation of the results, the normality of distribution was tested

using the Shapiro-Wilk W-test and the Kolmogorov-Smirnov test. The obtained data showed that the analyzed distribution did not differ from the normal one. This allowed us to use parametric criteria for statistical analysis of differences between samples for further analysis. The presence or absence of a linear relationship between two quantitative indicators, as well as their tightness and statistical significance, was determined by calculating the Pearson correlation criterion.

Represented scattering diagrams for the selected variables (Figures 5-7) indicated to the presence of a pronounced rectilinear dependence of the analyzed data. For neurologic symptoms, negative dependence was observed with reduced glutathione (r = -0.9877) and positive with nitrotyrosine (r = 0.9480). Consequently, the presence of a linear bond between nitrotyrosine and reduced glutathione (r = -0.9308) was natural. The calculated Pearson correlation coefficients were confirmed by visual 3D-diagrammma (Figure 8), that graphically shows the relationship between the dynamics of the concentrations of glutathione and nitrotyrosine with the severity of the neurological deficit.

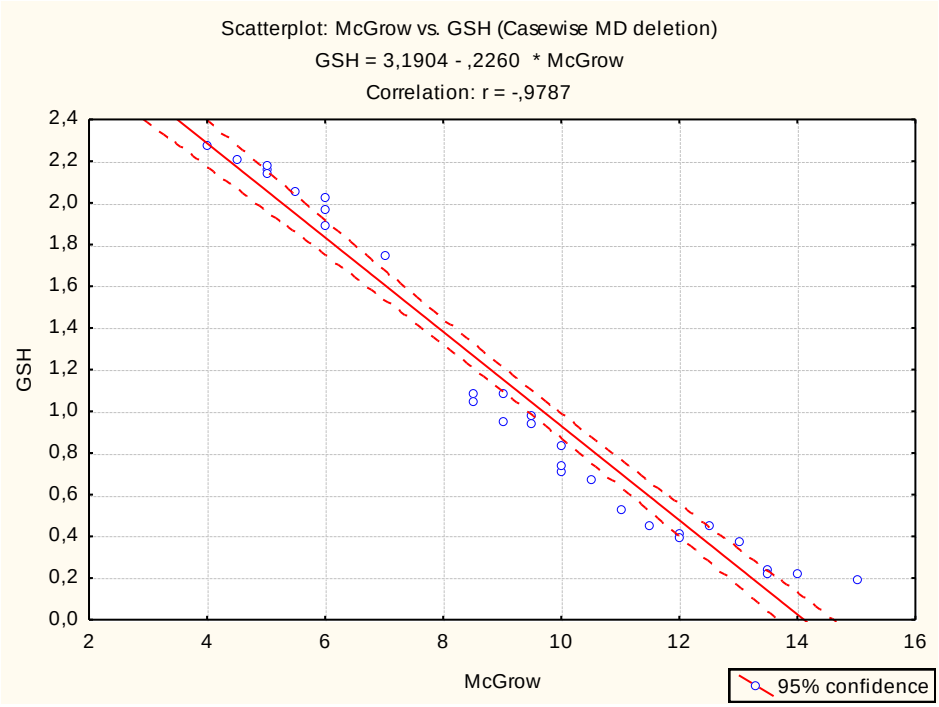

Figure 5. Dependence of stage of neurological deficit from reduced glutathione content in the brain of experimental animals

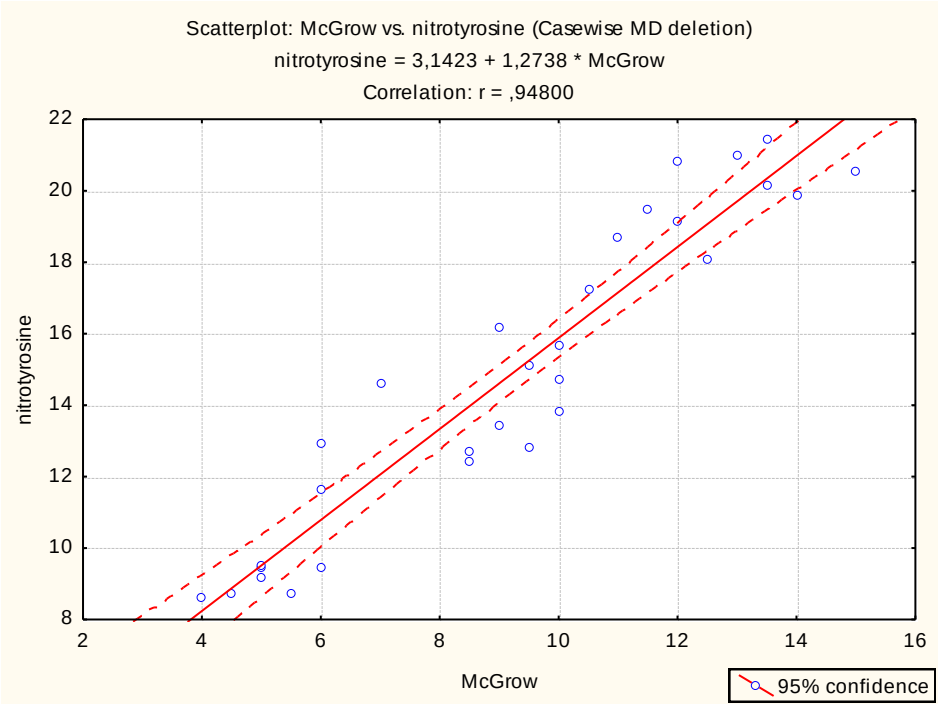

Figure 6. Dependence of stage of neurological deficit from nitrotyrosine content in the brain of experimental animals

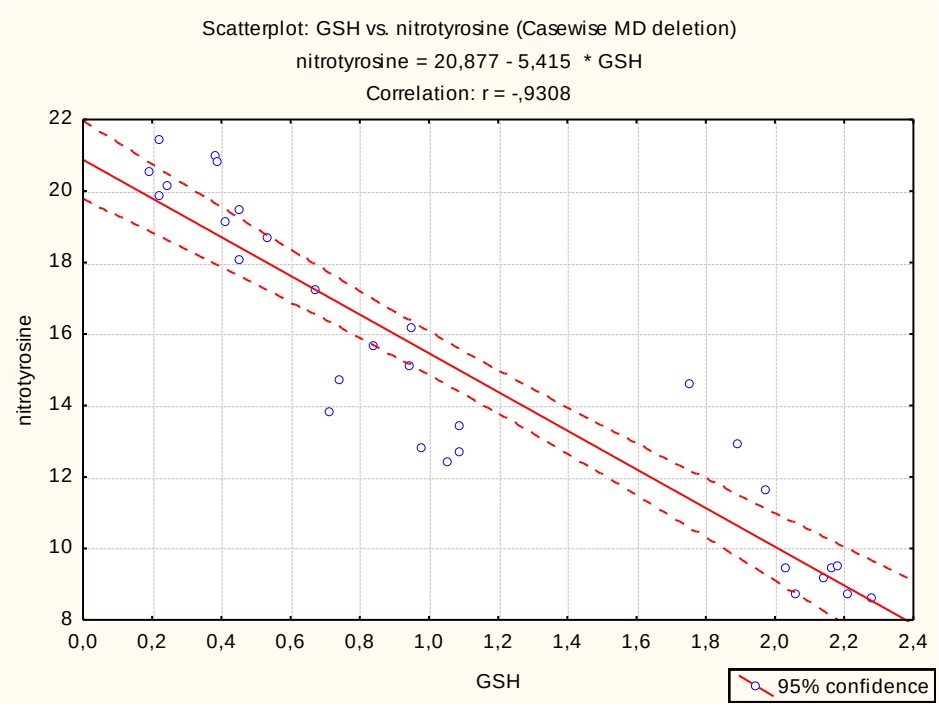

Figure 7. Dependence of nitrotyrosine concentration from reduced flutathione content in the brain of experimental animals

Although the correlation coefficient allows to get quantitative characterization of the degree of correlation of indicators, it is impossible to predict what will be the average of one indicator by the given value of the other. This problem was solved by applying regression analysis and calculating of coefficients of linear regression. During our calculating the linear regression, the neurological deficit was taken as a dependent variable. The results indicated about high accuracy of the linear model: the coefficient of multiple correlation R = 0.9731, the coefficient of determination R2 = 0.9469, corrected R2 = 0.943 at F = 241.01; Beta coefficient for glutathione = -0.5149 (p = 0.00023), Beta for nitrotyrosine = 0.4755 (p = 0.00054). Thus, the problem of regression analysis could be considered solved.

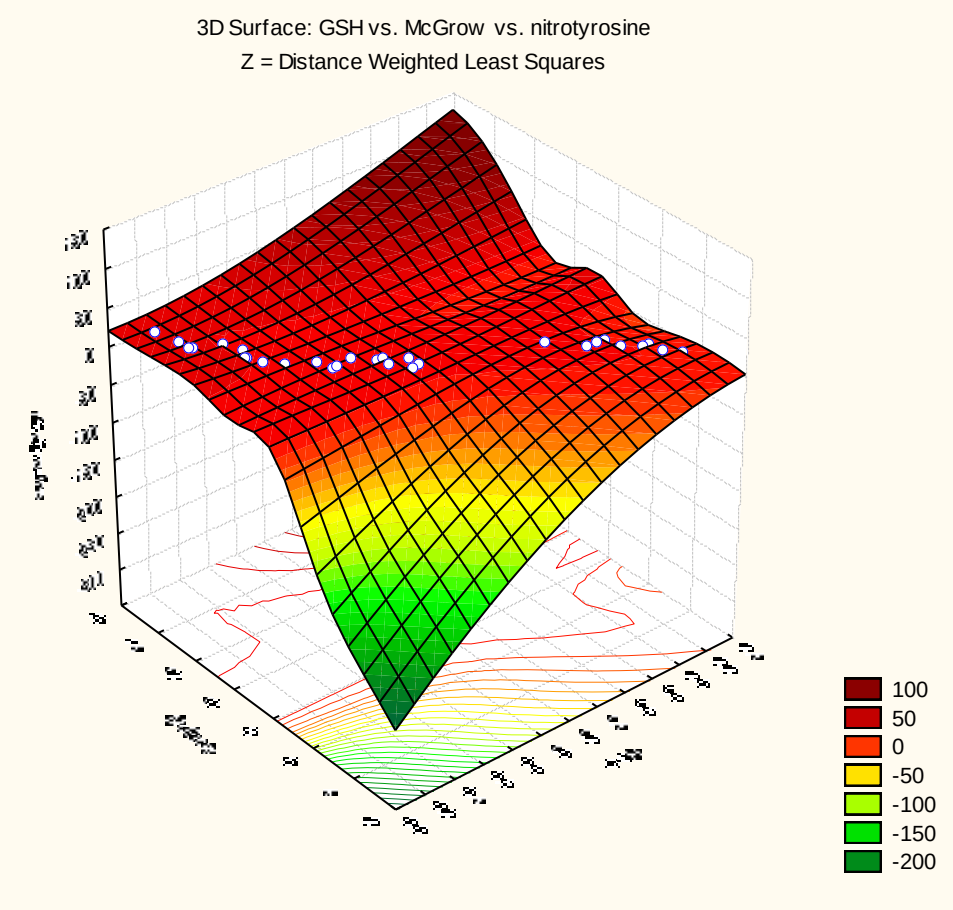

Figure 8. Conjugation of changes of nitrotyrosine and reduced glutathione levels in the brain tissue of animals with neurological deficit

The presence of a clear linear relationship between the studied indicators gave us ability to calculate the ratio cofficient of the level of nitrotyrosine to level

of reduced glutathione. We think, that this cofficient could be used in medical practice for the diagnostics of neurological disorders. Obtained data are represented in table 2. Thus, under normal conditions the ratio of nitrotyrosine / glutathione (NT/GSH) is about 1.3. Mild degree of neurological deficit is characterized by $K_{NT/GSH}$ close to 5.0; in severe neurological disorders, $K_{NT/GSH}$ increases and is about 65.0.

Thus, the conjugate system "nitric oxide - reduced thiols" plays an important role in the mechanisms of neuronal survival in conditions of ischemic neurodestruction. A key factor in maintaining of the normal functioning of the system is the keeping at a certain level of the pool of reduced thiols. They have transport properties to nitric oxide, regulate its bioavailability, and are scavengers of active forms of nitrogen and prevent the formation of peroxynitrite. There was revealed statistically significant linear dependence of the development of neurological deficit from the functioning of the conjugate system "nitric oxide - reduced thiols". The obtained results reveal the importance of the neuron glutathione system as an important target of neuroprotective therapy in ischemic stroke and are the experimental justification for the clinical application of thiol-disulphide system modulators. The coefficient of the ratio of nitrotyrosine to reduced glutathione can be used for the purpose of early laboratory diagnosis of cognitive-mnestic disorders in patients with cerebral pathology.

Thus, we identified specific markers for in vitro experiments, that had the greatest potential in providing information on some important mechanisms of neurotoxicity.

Table 2. Coefficient of nitrotyrosine/reduced glutathione levels ratio in groups of animals with different severity of neurological deficit

Group of animals	Coefficient nitrotyrosine/GSH
Intact (healthy animals)	1,345 ± 0,046
Group 1 (animals with mild neurological symptomatic)	5,08 ± 0,47
Group 2 (animals with middle degree of neurological symptomatic)	16,59 ± 1,43
Group 3 (animals with severe degree of neurological symptomatic)	65,4 ± 8,47

Discussion

Nowadays, use of in vitro tests in assessing safety during and new products drugs developing became common [9]. According to the principles of the US Government on the use of animals in research, testing and education, and the policy of the US Department of Health on the humane treatment and use of laboratory animals, in vitro tests for determining of basal cytotoxicity should be used before animal testing [10]. Such methods are considered part of approach for

estimation of the initial dose in the study of acute oral toxicity of drugs in vivo. For some of studied drugs, this approach reduces the number of used animals, as well as the proportion of animals that died during the experiment. A lot of large international companies have already widely used alternative methods. In 2007, the National Science Council of the United States published a report on the future of toxicological studies. The main proposal of this report was to reorient the testing to the molecular level instead of observing the phenotypic reactions of the whole organism (transition from testing on the whole animal to testing at the cellular level). Such proposals have serious reasons, because the imperfection and restrictions of testing on animals are obvious and understandable. Traditional toxicology in vivo is laborious, it takes a lot of time and consumes a large amount of the tested product. Animal testing is difficult to adapt to the current trend of high-performance screening technologies, that ultimately creates barriers to mass screening of drugs and chemicals. Nowadays, there is an urgent necessity to use of the latest scientific tools to prove the safety and efficiency of new products in the shortest time with greater reliability and lower costs [11, 12].

Nowadays it is possible to cultivate a wide range of cells of various types, tissues and species. This is very convenient, because could created conditions for determining the organo- and species-specific toxicity. In case of human cells usage, the problems of interspecific extrapolation are minimized [13].

Beside that, the use of cell cultures allows to establish the nature of the biological activity of the compounds directly at the cellular level and helps to account the complex synergistic and / or multidirectional effects of mixtures of chemical compounds. Major contribution to the development of a new field of toxicology was made by B. Ekwall [14, 15]. He formulated the concept of basic cytotoxicity, that is used for determination of the acute toxicity of chemicals on cell cultures instead of animals. In order to test this concept in practice, an international toxicology project was launched under the name of the Multicentre Evaluation of the In Vitro Cytotoxicity Program (MEIC). 50 selected chemical compounds were tested in 100 laboratories around the world, with more than 60 different in vitro tests. The results of the MEIC project clearly demonstrated the possibility of using in vitro tests to predict toxic concentrations of chemicals in the human body.

In the course of work on this project, three types of toxicity were identified [16]:

1. Basic (general) cytotoxicity - unfavorable effect of chemical compounds on the common for all cells structures and functions, that are necessary for cell survival, fission, DNA replication etc.

2. Organ-specific cytotoxicity - the effect on structures and functions, that are specific for certain cells of tissues and organs.

3. Extracellular toxicity - the xenobiotic does not directly affect on cell, but its effect is critical at the level of the whole organism and covers processes that occur outside the cells (for example, cellular secretion).

Toxicity studies in vitro are carried out on primary cultures of cells and tissues isolated from the body of animals, humans or individual types of tumors. Primary cultures of human cells include hepatocytes, epithelial cells, keratinocytes,

chondrocytes. Primary cultures of animal cells are represented by hepatocytes, blood cells. Among the permanent cell lines of animals in toxicological studies are used: mouse fibroblasts - L929, BALB 3T3; cells of hepatoma of rats - HTC; Ehrlich tumor cells of mice, etc. Among specialized cells are used peripheral blood erythrocytes of mice BALB/c, bull spermatozoa and rat muscle cells. Research on various cell lines provides clear information about the potential effects of drugs and their specific effects. Nowadays, studies of the total and specific toxicity of potential teratogens are most effective performed with the use of stem cells of human embryos that are capable to differentiating in vitro with the formation of myocardial, muscle, endothelial cells, hepatocytes. For evaluating tissue toxicity, tissue slices and reconstructed models are used that mimic normal tissues in vitro [16].

Today, commercial organotypic and reconstructed models of the epidermis are known, such as EPISKIN, EpiDerm, Full Thickness. These models are standardized, registered and submitted by the European Organization for Economic Cooperation and Development (OECD) in the list of instructions for testing the toxicity of chemicals, as well as in the new European legislation REACH [3, 17].

One of the most difficult fields in creating of alternative models for studying toxicity is the development of models of barrier systems of the body. In this direction the most significant success was achieved in the reproduction of the epithelial barrier, that is the first obstacle to the penetration of xenobiotics into the body. For Interstitial barrier studies, NT-29 and Saso-2 cell lines used. For the blood-brain barrier studies, which are important for many toxic substances, three-dimensional models containing glial and endothelial cells are being developed [19, 20].

Present methods of individual nerve cells obtaining make possible to get qualitatively new and reliable information about the state of the nervous tissue under conditions of testing of new chemical compounds or known drugs. For in vitro studies, white male rats are often used at the age of 4 weeks and weighing 80-100 g. The separation of enriched fractions of neurons and neuroglia is carried out in two stages. At the first stage, the brain tissue disintegrates in order to obtain a cell suspension; on the second stage, differential ultracentrifugation is used in a density gradient of sucrose and ficoll. To obtain neurons and neuroglia, the rats are decapitated and brain quickly extracted. Brain cortex separates from white matter, grinds up and transfer to solution, that contains 7,5% polyvinylpyrrolidone, 1% bovine serum albumin (BSA) and 10 mmole $CaCl_2$. Obtained suspension is filtered and layered on gradient, that contains 1 mole or 1.75 mole of sucrose. Centrifugation is carried out at 60000 g for 15 min (at 10 ° C). As a result of centrifugation, two layers and a dense precipitate are obtained. The upper layer is represented by the remains of myelin sheaths, the second layer contains glial and neuronal cells. The sediment is represented by neuronal bodies corresponded to a purity of 90%. Further purification of the second layer is carried out by secondary filtration and ultracentrifugation. The isolated neuronal cells are washed from sucrose and albumin with a cooled physiological solution (solution temperature 4

° C). Obtained suspension is divided into series: ***intact*** (suspension of neurons without application of the investigational pharmacological or chemical agents) and ***control*** (with addition to the suspension test substances).
Neurotoxicity of the test substances is evaluated by:

A) Test with silver nitrite. The principle of the method is based on the selective staining (in dark color) of the degenerating neurons. For this purpose, smears, that are made from a suspension of neurons of an intact, control and samples with the addition of potential neuroprotectors, are stained by silver nitrate to reveal degenerating neurons. Neurotoxicity of the test agents is assesed by the appearance of degenerating neurons in the sample;

B) The method of differential determination of apoptotic cells. For this the differential staining technique is used, that allows to stain apoptotic and necrotic cells by fluorescent dyes: Hoechst 33342 (apoptosis) and Ethidium bromide (necrosis). For this purpose, smears are stained for 15 min by Hoechst 33342 (50 μg / ml) and than by ethidium bromide (10 μg / ml). After each dye addition, the smears are washed with phosphate buffer, with further fixation in a 5% formaldehyde solution (20 min.). The number of necrotic cells is counted using a fluorescence microscope. Necrotic cells - large red cells, apoptotic - green with fragmented nuclei.

Suspension of neurons could also be used for study of number of biochemical indicators of energy metabolism, oxidative stress, NO-, GABA-ergic and thiol-disulphide systems, as well as some markers of destructive processes of the nervous tissue (neurospecific proteins, neurotrophic factors).

Study of neurotoxicity in vitro may be used of determinition of some molecular markers of the morphofunctional state of the nervous tissue [15, 16]:

1. The main myelin protein (MMP) is determined by immune enzyme analysis or by immunoblotting. MMP appears during any damage to the nervous tissue.

2. Phosphorylated neurofilament H (pNF-H) is determined by immune enzyme analysis or by immunoblotting. PNF-H is a sensitive marker of axonal injuries.

3. Expression / concentration of heat shock proteins in the brain is determined by immunohistochemistry, immunoblotting, immune enzyme analysis. HSP refers to highly conserved proteins and are found in all organisms from bacteria to humans. This indicates that they perform fundamental cellular functions. Both the cytoprotective properties of stress proteins and their role in the processes of normal cell vital activity are largely determined by the fact that these proteins are chaperones. Chaperones are proteins that facilitate the formation of the secondary and tertiary structure of other proteins. HSP is also involved in the processes of reparation or elimination of misfolded or denatured proteins. According to the modern classification, seven types of HSP are distinguished, that are divided either by molecular mass or by functions performed in the cell. There are small HSP (small HSP, s HSP) with molecular mass 25/27 kDa, 22 and 20 kDa, as well as high-molecular HSP 110, 100, 90, 70, 60, 40. HSP (like NO synthase) could be divided into the constitutional and inducible. Constitutive HSP

is synthesized in the cell all the time, and activation of them does not require exposure to a damaging factor cell, i.e. their synthesis under stress does not increase. Synthesis of inducible HSP begins after exposure to the cell of the damaging agent.

4. DR5 (Death Receptor) is determined by immunohistochemistry, immunoblotting. DR contain the cytoplasmic death domains of DD (death domain), that after linking to the death ligand, attract the adapter proteins containing the DD domains and the death effector domain (DED).

5. The family of Bcl-2 is determined by immune enzyme analysis, immunohistochemistry, immunoblotting. The family of cellular proteins Bcl-2 has 17 members. These proteins exhibit a wide range of activity from inhibition of apoptosis to its induction. The family includes subfamilies that differ by functionally and structurally: the subfamily of the closest homologues of Bcl-2 (Bcl-2, Bcl-XL, Bcl-w, etc.) - inhibitors of apoptosis. Proteins of the subfamilies Bax and BH3 - are promotors of apoptosis. Apoptosis is associated with various changes in mitochondria, including the release of cytochrome-C into the cytoplasm. Bcl-2-related proteins are involved in the regulation of these changes by forming channels in the membrane through which cytochrome-C enters the cytoplasm. In this case, Bcl-2 and Bcl-XL inhibit the release of cytochrome-C, and Bax-stimulates. However, Bcl-2 can inhibit the ability of Bax to form channels. In addition, Bcl-2 and Bcl-XL can bind cytochrome-C directly and displace it from the apoptosome, that prevents caspase activation.

Its well known, that mitochondria is a priority target in the implementation of the toxicodynamics of many drugs (cytostatics, antibiotics, cardiac glycosides, etc). So, it is useful to assess the "mitodestructive effect" in toxicological studies in vitro [15].

For this study the mitochondrial fraction of brain cells is used. Pieces of the brain are placed in liquid nitrogen, grounded to a powdery state and homogenized in 1000 % w/vol of the medium consisting of: sucrose – 250 mM, Tris-HCl-buffer – 20 mM (pH 7.4) and EDTA – 1 mM. Mitochondria are isolated at 4°C by differential centrifugation in the refrigerated centrifuge Sigma 3-30k (Germany). For cleaning the mitochondrial fraction from large cell fragments primary centrifugation is conducted for 7 minutes at 1000 g, and then supernatant is centrifuged for 20 minutes at 17000g. The supernatant is decanted and stored at -80°C. The pellet of mitochondria is resuspended in the medium, containing bovine serum albumin (0.5 mg/ml) and then precipitated by centrifugation for 10 minutes at 17,000 g. The mitochondria are suspended in the isolation medium, suspension contained 40-60 mg protein/ml. To record the opening of mitochondrial oscula 1 mg of mitochondria suspension is used, that is added to incubation mixture.

Variants of the incubation mixture:

1. 120 μM of KCl, 0.5 mM of KH2PO4, 2 mM of glutamate, 1 mM of malate and 20 mM of Tris-HCl-buffer (pH 7.4).

2. 70 μM sucrose, 5 μM HEPES, 70 μM KCl, 0,5 – 1 μM KH_2PO_4, pH 7,4.

The total volume of the incubation mixture is 3 ml.

Before the experiment, the test substances (10-100 μM in the sample) are added into the samples and then incubated for 2 minutes.

The mitodestructive effect is studied by the ability of the substance to enhance the opening of the mitochondrial oscula and to reduce the mitochondrial potential Ψ.

Conclusion

Thus, studies have shown that the reconstructed models that mimic normal tissues in vitro, had a good correlation with the systemic toxic effect, that determined in animals. So, studies with usage of cell cultures allow a comparative rapid assessment of the toxicity and hazards of a chemicals. They also allow to study metabolic abnormalities in the cell, cytotoxic and organotoxic, mutagenic and carcinogenic effects.

To study the cytotoxicity of xenobiotics on cell culture, the following tests are recommended:

1) Determination of the number of viable cells using trypan blue;

2) Determination of the total protein content as an indicator of cell mass increase, that may also be an indicator of cell proliferation;

3) Determination of changes of respiratory enzymes activity in the test with methyl tetrazolium;

4) Assessment of lysosomal activity and intensity of active membrane transport processes - by absorption of neutral red dye;

5) Assessment of the degree of damage of cytoplasmic membrane - by lactate dehydrogenase (LDH) activity.

To study organotoxicity, the same tests are used, as well as specific markers: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) for liver cells; LDH and troponin - for cells of cardiac tissue, rate of collagen synthesis - for skin fibroblasts, rate of synthesis of interleukins and antibodies, C-reactive protein content - for immunotoxicity.

In addition, for all types of cells, PCR (polymerase chain reaction) can be used to determine the activation of a gene of some protein, enzyme, cofactor. The next parameters are defined based on named above tests: IC50 (concentration, inhibiting the growth of 50% of cells in culture), LC50 (concentration of substance causing death of 50% of cells), EC50 (concentration causing a decrease in function of 50% of cells).

Experiments on cell culture in vitro have several advantages in comparison with animal tests. First, cell culture tests can be carried out in microquantities. The dose of the test substance obtained by each cell can be measured and monitored with high accuracy, that facilitates the determination of toxic concentration of xenobiotic. Secondly, studies on cell culture yield results that allow to study «dose-effect» and «structure-activity» relationships. Third, cell culture method allows the screening of several substances simultaneously and directly on the cells of human organs.

The problem of introducing alternative methods for studying the toxicity and safety of various chemicals into the system of preclinical research remains relevant, but at the same time is still far from complete. Widespread implementation of in vitro methods can only be realized as a result of united efforts of toxicologists and representatives of government agencies.

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