

LONG-TERM RESULTS OF PHARMACOLOGICAL CORRECTION OF INOS, ENOS, NNOS MRNA EXPRESSION DISORDERS IN RAT HIPPOCAMPUS AFTER CHRONIC PRENATAL HYPOXIA

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Chronic prenatal hypoxia (CPH) is the main damaging factor in fetal nervous system lesion and the subsequent occurrence of the risk of the psychomotor and somatic disorders formation, often not compensated for the whole subsequent life. The possible complications of the child development in the womb, as well as his future health, depend on the timing when hypoxia occurs and its course [1,2,3].

Lack of oxygen during critical periods of brain development leads to biochemical and structural changes in the developing brain, and, as a consequence, to the pathological development of the fetal brain. Being one of the main causes of mortality and neurological disability, hypoxic brain disorders have a significant impact on many individual characteristics of the physical and intellectual spheres of the developing organism [4].

Hypoxia causes a violation of oxidative processes, a decrease in the cellular energy balance, an excess of neurotransmitters, and glia and neurons metabolism alterations. Under hypoxia, lipid peroxidation becomes activated with the accumulation of aggressive free radicals, which have a destructive effect on the membranes of neurons [5].

Nitric oxide (NO) is one of the most important biologically active substances involved in many physiological and pathophysiological processes. Possessing a wide spectrum of bioregulatory activity, NO plays an important role in the physiology of various cells, [6, 7].

Nitric oxide is a gas neurotransmitter of the nervous system. Due to physicochemical characteristics, this molecule can freely migrate through cell membranes, acting as an intracellular and intercellular signaling molecule. Nitric oxide is synthesized from L-arginine by NO synthase [8, 9].

As an intercellular and intracellular messenger, NO participates in the regulation of various metabolic reactions that ensure the viability and functional activity of cells and the organism as a whole, but under certain conditions it may be involved in pathological processes. Targets of NO exposure depend on environmental conditions and the amount of NO produced. The local level of NO is determined by the balance between the intensity of its synthesis or exogenous production and the intensity of inactivation. The physiological effect of NO varies from modulation of the vascular system functioning to the regulation of immune processes and control of neuronal functions (signal transmission in non-adrenergic non-cholinergic neurons, synaptic plasticity in the central nervous system, oscillatory activity of the neuronal network, neuroprotection) [9, 10].

The NO synthesis is carried out with the participation of NO synthase, which has three isoforms: neuronal (nNOS, NOS-1), inducible (iNOS, NOS-2) and endothelial (eNOS, NOS-3). Enzymes catalyze the five-step oxidation of L-arginine to L-citrulline and NO. NO synthases are homodimers with a molecular mass of 130 kDa for iNOS and eNOS, 160 kDa for nNOS [9, 11]. Constitutive isoforms of NO synthase are constantly present in the corresponding cells, they are associated with their membrane proteins and have predominantly physiological significance, since the amount of nitric oxide formed by them

is relatively small [12]. Neuronal NOS acts in the regulation of growth and differentiation of central nervous system (CNS) cells and, presumably, in their recovery after local ischemic brain damage [9, 13]. Endothelial NOS plays a leading role in ensuring a constant baseline level of NO, which is associated with the implementation of local endothelial cytoprotection mechanisms and the maintenance of vascular homeostasis [3, 9]. A decrease in the activity of this isoform leads to an increase in endogenous NO deficiency and is one of the key factors in cerebrovascular pathology. nNOS and eNOS are Ca²⁺ dependent. Neuronal and endothelial NO synthases are constitutive enzymes, the activation of which, as a rule, is associated with immediate allosteric modulations of the enzyme molecules, in contrast to iNOS, whose activity increases at a longer time period due to activation of the corresponding genes expression. iNOS is not associated with membrane proteins. It is synthesized in the cytosol in response to an external influence on the cell and demonstrates activity 6-8 hours after external exposure. Activation of iNOS induces the NO synthesis in high concentrations, which can be toxic to cells [14]. Recently, another constitutive form of NOS was found out in mitochondria, which was identified as mtNOS. It is mainly localized in the mitochondria of the brain [15]. Mitochondrial NOS (mtNOS) is similar in structure and functional features to inducible (iNOS). Nitric oxide synthesized by the mitochondrial isoform of NOS regulates mitochondrial activity and redox homeostasis. However, the question of what mtNOS is - a separate isoform of the enzyme or inducible NOS modified during translation or after, remains open. Low concentrations of NO have a neuroprotective effect in the brain, and relatively high concentrations arising from intense excitation contribute to neurons damage [3, 4, 7, 9]. CPH influence causes the activation of constitutional NO synthases as a compensatory reaction at the first stages. Further, the expression of iNOS in neurons, endothelial cells, activated astrocytes and microglia cells increases. The implementation of the research achievements of the nitroxydergic system under the CPH into practical medicine is fraught with a number of difficulties, the main one is the insufficient knowledge of the nitroxydergic system disorders in brain pathology after chronic exposure to hypoxia, as well as the almost complete absence of pharmacological agents that selectively affect the production or metabolism of NO in the body. Nevertheless, an active search for drugs that can influence developing pathological processes and can affect the activity of NO synthases is carried out [16]. Unfortunately, there is no common concept of neuroprotection following CPH to date. We have outlined several promising links of neuroprotection targets for this pathology: imbalance of coupled NOS systems, ROS overproduction, depression of endogenous neuroprotectors HSP70 / HIF-1 α , SERM modulation, neuroapoptosis. A nitroxydergic system, namely iNOS, can be considered as an upcoming target for neuroprotection after exposure to CPH. We selected drugs demonstrating anti-ischemic, antioxidant, endothelioprotective activity, affecting the expression of cytoprotective proteins and in which neuroprotective activity was predicted - antioxidants Thiotriazolin and Mexidol, NO mechanisms modulator - L-arginine, modulators of the glutathione system (HSP - systems) - Glutoredoxin and Angiolin, selective estrogen receptors modulators - Tamoxifen, neurometabolic stimulant - Piracetam [17,18,19, 20]. Of greatest interest in this regard is (S) -2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate "Angiolin" synthesized by NPC Farmatron (RF Patent 2370492, IPC C 07 D 413/00 (2006.01)) [21]. The use of Angiolin in case of acute cerebral ischemia has led to an increase in the survival of sensorimotor cortical neurons and cerebral vascular endotheliocytes, increase in VEGF expression, and normalization of the NO / SH ratio [20].

The aim of this work was a comparative analysis of the long-term effects of pharmacological correction of nitroxydergic system disorders in the hippocampus of rats after CPH using well-known neuroprotective drugs and the new original drug "Angiolin".

Materials and Methods. The experimental part of the study was carried out in strict accordance with the national "Joint Ethical Principles of Animal Experiments" (Ukraine,

2001), consistent with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (Council Directive 2010 / 63EU of the European Parliament and of the Council (September 22, 2010) on the protection of animals used for scientific purposes). The studies were done on 36 male 1.5- month-old Wistar rats, obtained from females who were undergone modeling of chronic prenatal hypoxia in the progeny from the 16th day of pregnancy. For this purpose, we used the model of chronic hematic nitrite-induced prenatal hypoxia [21-24] in our modification. Pregnant female rats were daily injected subcutaneously with sodium nitrite at a dose of 50 mg / kg (dose causing moderate hypoxia) from 17 to 21 days of pregnancy (corresponding to the third trimester of pregnancy). Pregnancy term was determined counting from the date of sperms detection in a vaginal smear.

Newborn animals were divided into 9 groups: 1st — intact animals obtained from females with normal physiological pregnancy, 2nd group — control animals after simulated CPH, which were injected with 1 ml of physiological saline; 3–9 groups - animals after PH, to which, after birth, the drug was administered intraperitoneally in an effective dose (see table 1)

Table 1.**Drugs used in the experiment.**

No gr.	Drug (manufacturer)	Dose
2	Physiological saline	1 ml
3	L-arginine (LLC Elite-Farm)	200mg/kg
4	Tamoxifen (SALUTAS FARMA / SALUTAS PHARMA GMBH)	0,1 mg/kg
5	Piracetam (JSC «Galichpharm»)	500 mg/kg
6	Angioline substance obtained at the DP "Plant of Chemical Reagents", Kharkov (quality certificate No. 1, series No. 010713), a 2.5% injection solution was prepared at NPC Farmatron, Zaporizhia)	50 mg/kg
7	Glutoredoxin (Grx-1) (Sigma-Aldrich)	200 µl/kg
8	Thiotriazolin (JSC Galichpharm. Created by NPC Farmatron)	50 mg/kg
9	Mexidol (MC ELLARA)	100 mg/kg

A study of NOS isoforms mRNA expression was conducted at the Department of Molecular Genetic Research of the Educational Medical and Laboratory Center of ZSMU. In order to assess the state of NOS mRNA expression, the real-time reverse transcription polymerase chain reaction (RT-PCR) was used. Molecular genetic research included several stages. Tissues were dewaxed by incubation in two consequent xylene baths for 5 minutes each, and then in two consecutive baths of 100% ethanol for 5 minutes each. After wax removal and centrifugation, the precipitate was dried in air to remove residual ethanol. Isolation of total RNA from rat tissue was performed using the Trizol RNA Prep 100 kit (IZOGEN, Russia), which contains the following reagents: Trizol reagent and ExtraGene E. RNA was isolated according to the kit protocol. For reverse transcription (synthesis to DNA), we used the "Reagent kit for reverse transcription (OT-1)" (SINTOL, Moscow). The preparation and conduct of the reaction was carried out according to the kit protocol. To determine the level of the studied genes expression, the CFX96™ Real-Time PCR Detection Systems (Bio-Rad Laboratories, Inc., USA) amplifier and a set of reagents for RT-PCR in the presence of SYBR Green R-402 (SINTOL, Moscow) were used. The final reaction of the amplification mixture included SYBR Green dye, SynTaq DNA polymerase with antibodies that inhibit enzyme activity, 0.2 µl of direct and reverse specific primers, dNTP-desoxynucleoside triphosphates, 1 µl of matrix (cDNA). The reaction mixture was brought to a total volume of 25 µl by the addition of deionized H₂O. Specific primer pairs (5'-3') for analysis of the studied and reference genes were

selected using PrimerBlast software (www.ncbi.nlm.nih.gov/tools/primer-blast) and manufactured by ThermoScientific, USA.

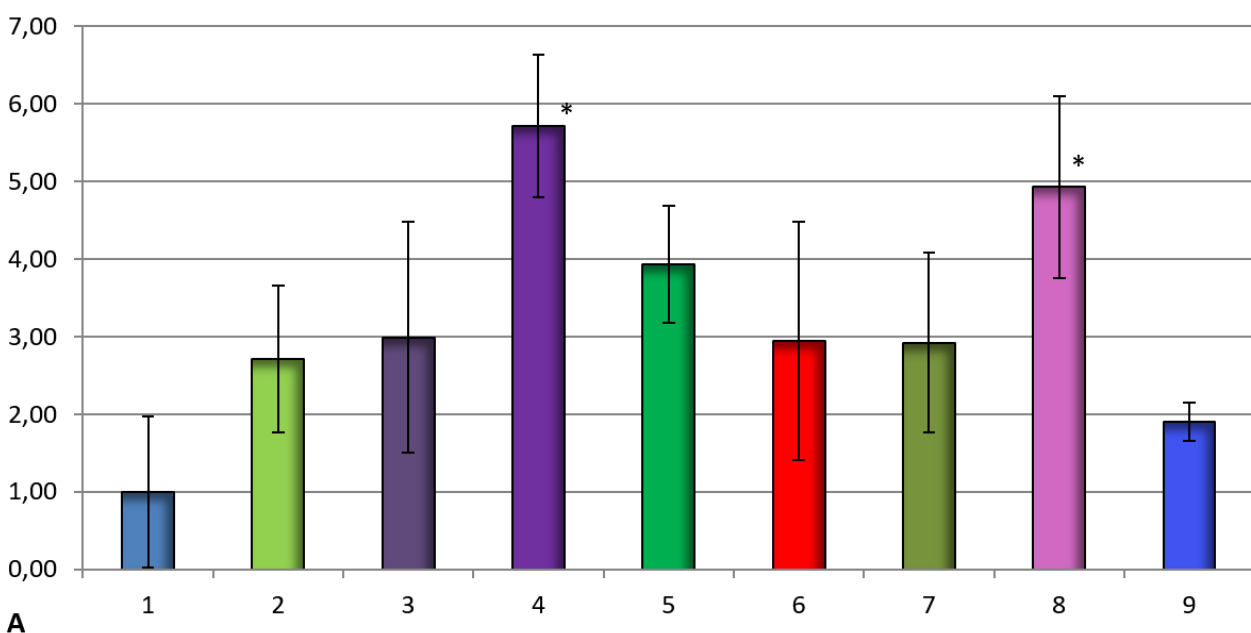
Table 2.

Specific primers used in real time RT-PCR.

Gene	Nucleotide sequence of the primer	T _m , °C	PCR product length, bp	Exon-exon junction
NOS1	F = GACGCAGATGAGGTTTTTCAGC R = GGGGGCAGGAGGATCCAG	59.87 61.17	45	4477/4478
NOS2	F = GTCCTCAGGCTTGGGTCTT R = CCGTGGGGCTTGTAGTTGAC	59.6 60.95	49	143/144
NOS3	F = CCCAGGAGAGATCCACCTCA R = CAGCACATCCTGGGTCTGT	60.03 59.96	58	2899/2900

Amplification occurred under the following conditions: initiated denaturation 95 ° C for 10 min.; further 50 cycles: denaturation - 95 ° C, 15 sec., annealing of primers - 58-63 ° C, 30 sec., elongation -72 ° C, 30 sec. The fluorescence intensity was automatically recorded at the end of the elongation stage of each cycle via the SybrGreen channel. The actin, beta gene (*Actb*) was used as a reference gene to determine the relative value of the change in the studied genes expression level. Significance of differences between the experimental groups was performed using the nonparametric Mann-Whitney U-test. Differences with a significance level of more than 95% ($p < 0.05$) were considered reliable. The research results were processed using the statistical package of the licensed program "STATISTICA for Windows 6.1"

Results. As a result of a study of mRNA expression by PCR, it was found that the modeling of CPH leads to persistent impairment of iNOS, eNOS, nNOS mRNA expression in 1.5-month-old experimental animals. Thus, the expression of nNOS mRNA is increased by more than 2 times, the expression of iNOS mRNA rises in 4 times in comparison with the intact group, and the expression of eNOS mRNA is reduced in 2 times. We have found that the use of L-arginine, Angiotensin and Glutaredoxin did not lead to significant changes in the expression of nNOS mRNA, in comparison with the control group. In groups of animals after CPH and the administration of Tamoxifen and Thiothiazolin, the level of nNOS mRNA expression significantly exceeds the control indices (by 110% and 81%, respectively ($p < 0.05$)). The rate of nNOS mRNA expression in the group of animals after CPH and the injection of the Mexidol is 30% lower than in the control group.



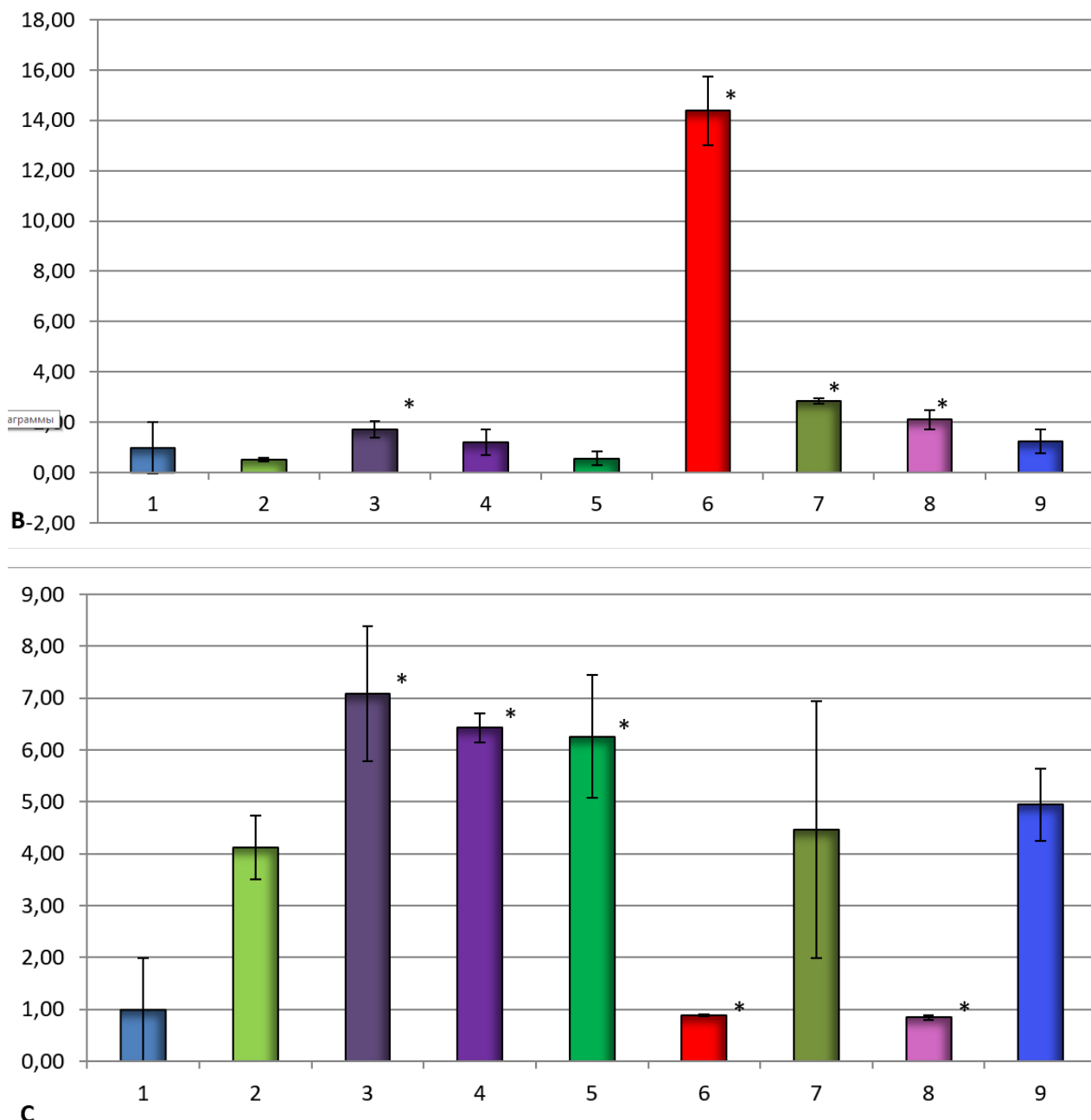


Fig. 1. Relative normalized expression of mRNA of (A) nNOS, (B) eNOS, (C) iNOS (1 - group of intact animals, 2 - control group after CPH, 3 - after CPH and injection of L-arginine, 4 - Tamoxifen, 5 - Piracetam, 6 - Angiolin, 7 - Glutoredoxin, 8 - Thiotriazolin, 9 - Mexidol).

Note: * - significant differences in parameters ($p < 0.05$) in relation to the control group.

Studies of the expression of eNOS mRNA showed that in all groups except animals treated with Piracetam, the level of eNOS synthesis was higher than the control values (in 2.3 times after the injection of Tamoxifen and Mexidol, in 3.3 times for L-arginine, in 4 times for Thiotriazoline, in 5.5 times for Glutoredoxin and in 27.6 times for Angioline). The expression of eNOS mRNA in animals after injection of Angioline was significantly ($p < 0.05$) higher than that of all experimental groups. The level of eNOS mRNA expression in animals treated with Piracetam does not differ from the control indices.

The nature of the iNOS mRNA expression in groups of animals after CPH treated with the studied drugs demonstrates different directions of their actions. So, in animals, after the injection of Glutoredoxin and Mexidol, the level of expression of iNOS mRNA ap-

proaches the control values. The iNOS mRNA content in the groups of animals treated with L-arginine, Tamoxifen, and Piracetam was significantly higher than the control group value (72%, 56%, and 52%, respectively ($p < 0.05$)). Only two drugs - Angiolin and Thio-triazolin demonstrate a significant decrease in iNOS synthesis in 5 times in comparison with the control and are significantly comparable with the indices of the intact group.

Studies have shown that in 1.5-month-old rats undergoing CPH, the expression character of NOS mRNA in the CA1 zone of the hippocampus changes - an increase in the expression of nNOS mRNA and iNOS mRNA in response to activation of oxidative stress and neuroinflammation takes place [20]. Pharmacological correction of simulated CPH changes the character of mRNA expression of all NO synthases isoforms.

Determination of mRNA expression is a modern highly informative method for evaluation of the structure functional activity, activation of the corresponding genes, and intracellular synthetic processes. Increased mRNA expression directly indicates the activation of certain genes of the cell genome. This activation is associated with action of stimulating signals for activation of adaptive-compensatory intracellular mechanisms. The obtained data are in line with the modern concept of neurodegeneration in cerebral ischemia, which is consistent with our previous studies, demonstrating a significant increase in NO production due to increased activity and expression on account of nNOS and, especially, iNOS [17-20]. Also, we found inhibition of eNOS mRNA expression in the hippocampus CA1 zone of 1.5-month-old rats undergoing CPH. This does not contradict the data of other researchers and the ideas about the cascade mechanism of ischemic neurodegeneration, which shows an increase in the expression of iNOS and nNOS [2, 6, 7, 9, 11, 13, 16]. The activity and expression of eNOS may vary with the time and severity of ischemic brain damage. In the first minutes after ischemia, activation of eNOS expression takes place. It is associated with activation of c-fos and VEGF, and in the longer term of the pathology, this constitutional iso-enzyme is inhibited in response to activation of oxidative stress and neuroinflammation [6, 20, 25, 27].

Neuroprotection or neurotoxicity of nitric oxide produced by various isoforms of NO synthases after the damaging effects of CPH is determined by both the stage of the process and the depth of brain tissue damage [25]. There are no data in the literature on the character of changes in the expression of NO synthases in the brain after CPH. The available data on disorders in the nitroxydergic system after acute hypoxia and ischemia demonstrate the staged nature of these changes. According to Viktorov I.V. (2000) in the initial period, the expression of constitutional NO synthases, aimed at compensating for ischemia, predominates. Next, the production of nitric oxide and other free radicals increases sharply, giving rise to intense oxidative stress. Since the main mass of neurons (about 98%) does not contain nNOS, its damage is determined by exogenous nitric oxide, the sources of which, in addition to endotheliocytes, are NOS neurons, activated astrocytes containing both iNOS and nNOS, microglia. It has been suggested that at the initial stage of occurrence of ischemia, the activation of the nitroxydergic system can have a beneficial effect due to an increase of cerebral blood flow, but in a later period it causes a neurotoxic effect associated with an increase of free radical processes [27, 28]. Excessive production of NO can lead to excitotoxicity, apoptosis, and inflammation. Van den Tweel E.R (2005) has shown that combined inhibition of neuronal nitric oxide synthase (nNOS) and inducible NOS (iNOS) can reduce hypoxia-ischemia-induced brain damage in 12-day-old rats [29]. However, inhibition of eNOS is harmful, as it leads to a decrease of cerebral blood flow and to increase of hypoxic effects.

Currently great efforts are applied to research and development of drugs - selective inhibitors of nNOS, the effects of which would not affect the synthesis of eNOS [30, 31]. Regarding inhibition of iNOS, opinions are controversial.

Some authors point to the need to reduce the level of iNOS [31,32]. Other authors argue that iNOS is necessary to manage balance between relaxation and constriction factors in the cardiovascular system which is a subject to chronic hypoxia [33].

Xingping Q. (2019) indicates that hypothermia, which is used globally as the main

therapeutic tool for the treatment of CPH, prevents the increased expression of nNOS and iNOS in the cerebral cortex and thereby prevents an increase in NO and reduces damage to neurons [34].

The data obtained by us correspond to modern concepts of the brain nervous tissue nitroergic system disorders after hypoxic exposure [4, 5, 12, 35]. Analysis of the results of the prolonged effect of the studied drugs on the level of expression of mRNA NO synthase isoforms shows that the use of the studied drugs led to normalization of iNOS, nNOS mRNA expression in animals exposed to CPH. Actively reducing mRNA nNOS expression were the following drugs: Mexidol, Angiolin, Glutoredoxin and L-arginine. Effects of Piracetam and Thiotriazolin on reduction of the neuronal NOS synthesis were, in comparison with other drugs, less effective.

In the results of the examined drugs' effect on reducing the level of inducible NOS expression, Angiolin and Thiotriazolin have been more effective drugs, and L-arginine, Tamoxifen and Piracetam have shown a more moderate therapeutic effect.

Expression of mRNA of the NOS endothelial isoform, according to the literature, demonstrates a contradictory nature. According to the numerous data, eNOS is activated at the beginning of hypoxic exposure, and at chronic oxygen deficiency its synthesis decreases [9, 11, 13]. Wang H. et al. (2009) found that eNOS expression was reduced after OGD reperfusion injury. Yagita et al., 2013 showed that eNOS dysfunction leads to endothelial dysfunction and inhibits tissue repair after ischemic injury [37].

The results of the studied drugs effect on the level of eNOS mRNA expression demonstrate the undisputed leader in stimulating the synthesis of endothelial NOS - the drug Angiolin. The remaining drugs have also increased the level of eNOS synthesis in comparison with the control in 2-5 times. A comparative analysis of the therapeutic effect of L-arginine, Tamoxifen, Piracetam, Angiolin, Glutoredoxin, Thiotriazolin and Mexidol on the recovery processes after PH indicates the most effective impact on the dynamics of all NOS isoforms the synthesis of the drug Angiolin.

An increase in the expression of endothelial NOS, normalization of the neuronal NOS and a decrease in the inducible NOS expression can be regarded as a manifestation of the endothelial and neuroprotective effects of the drugs. Considering a similar effect in the studied drugs, we can conclude not only about their effectiveness, but also the correctness of the choice of the target link with CPH. The most effective was the effect on the target link of ischemic brain damage in chronic renal failure, such as a violation in the conjugated NO / SH system by thiol antioxidants by ROS and NO scavengers, Thiotriazolin and, especially Angiolin.

Thus, the revealed decrease of iNOS mRNA expression under the influence of Angiolin and Thiotriazolin can be explained by its ability to reduce the level of ROS and cytotoxic forms of NO participating in the regulation of this enzyme expression [35, 38]. The decrease in iNOS mRNA expression can also be explained by the property of the Angiolin and Thiotriazolin molecule fragment, namely, the properties of 3-methyl-1,2,4-triazolyl-5-thioacetate to bind excessive ROS and protect sensitive cysteine residues - Cys 252, Cys 154 and Cys 61 in its DNA-binding domains, and to participate in the reduction of these groups in a reversible inactivation, playing the role of Redox Faktor-1 [35]. Reducing the excessive level of ROS, Angiolin and Thiotriazolin, indirectly through regulation of the transcription factor NF- κ B, are able to regulate the expression of redox-sensitive genes, including those responsible for iNOS synthesis [35]. The higher Angiolin activity compared to Thiotriazolin can be explained by the presence of L-lysine residue in its structure, which can interrupt ROS-dependent mechanisms of IL-1 β and TNF- α expression, as well as increase in the bioavailability of L-arginine [38]. Of interests are the mitoprotective properties of Angiolin and its ability to reduce ROS by mitochondrial bioenergetic reactions also. If the fragment of the Angiolin molecule - 3-methyl-1,2,4-triazolyl-5-thioacetate is able to influence on compensatory cytosolic mitochondrial shunts, the L-lysine residue together with vitamin C can form L-carnitine

[39]. Also, the L-lysine residue can affect the expression of eNOS, and Angiolin is able to increase the expression of VEGF in the capillaries and vessels of the muscle type in the brain [20]. The effect on transcription processes noted for Angiolin can be explained by its effect on c-fos and HSP70 [40]. The obtained effects of Mexidol are also explained in terms of the antioxidant properties of its structure —oxypyridine, which binds free radicals, increases glutathioneperoxidase activity, and the ability of the succinic acid in the Mexidol molecule to reduce the formation of ROS by mitochondrial bioenergetic reactions [20, 41]. Firstly identified properties of Tamoxifen to regulate the expression of NOS isoforms mRNA are explained not only by its ability to increase the level of intramitochondrial HSP70 after interaction with ER_β and regulate ROS levels, but also by the direct antioxidant properties of this drug [20, 42]. In addition, at the examined dose, Tamoxifen modulates ER_β, by regulation of hormone-activating transcription factors which initiate gene transcription [42]. The therapeutic effect of L-arginine is directly related to the production of NO and its antioxidant properties [43]. However, the low efficiency of L-arginine is explained by a sharp decrease in NO bioavailability under the conditions of oxidative stress, and by the loss of its antioxidant and signaling properties, by the conversion of NO to the cytotoxic ONOO- or nitrosonium ion [20]. In this regard, the question of the combined use of L-arginine with thiol antioxidants that increase the NO bioavailability due to the formation of nitrosothiols is considered [20, 43, 44].

Conclusion. The nitroergic system, especially its link such as expression of mRNA of nNOS, eNOS and iNOS, is an important link - the target of neuroprotection in CPH. Pre-selection of drugs - the candidates – antioxidants received experimental basis. The thiol scavengers ROS / NO – Thiotriazolin and Angiolin have shown the greatest efficiency in regulating of the expression of nNOS, eNOS, iNOS mRNA in animal CA1 hippocampus after CPH. The new original drug Angiolin, possessing endothelioprotective and neuroprotective effects, can be considered as a promising agent for correction of the CPH negative effects in newborns.

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Ethical considerations

All investigations conformed to the ethical of research and were approved by the Bioethics Committee of Zaporizhzhia State Medical University.

Conflict of interest

All authors declare that no conflict of interest exists.

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