

Reduce the Neuroapoptosis in the Brain of Rats Born to Mothers with Experimental Placental Insufficiency by Combination of Thiotriazoline with L-Arginine and Thiotriazolol with Piracetam

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Abstract

Our study experimentally provides promising opportunities for further clinical use of a combination of the essential amino acid L-arginine with a thiotriazoline in placental insufficiency (PI). The biological functions of L-arginine are substantiated by the fact that it is a precursor to the synthesis of nitric oxide (NO). Thiotriazolol is able to act as a transport molecule NO, forming nitrosothiols. The pharmacological effect of the combination is due to the mutual potentiality of thiotriazoline and arginine on the synthesis, transport and bioavailability of NO and the physiological functions of this molecular messenger.

Keywords: Placental insufficiency; Nitric oxide; Argitril; Thioacetam; Neuroapoptosis

Introduction

An important task of modern experimental pharmacology is the search for new perspective drugs for treatment complications of the pregnancy. There is a reduction in the number of normal births to 15.0-20.0% in modern conditions. Miscarriage occurs in 10.0-25.0% of pregnant women, resulting in 5.0-10.0% preterm labor [1].

At the same time, the share of premature infants accounts for 70-80% of the early neonatal mortality and to 60.0-70.0% of all cases of childhood mortality. Most of the pathological births and complicated pregnancies are the result of primary and secondary morphofunctional disorders in the fetoplacental system [1,2]. PI is diagnosed in 3-4% of healthy women with uncomplicated pregnancy, and with different pathologies its frequency increases to 24-46% [2].

In the development of placental insufficiency there are several interrelated pathogenetic mechanisms: insufficiency of cytotrophoblast invasion, pathological changes in utero-placental circulation, violation of fetoplacental blood flow, lesion of the placental barrier with violation of its permeability, reduction of compensatory and adaptive reactions in the mother-placenta system-fetus.

It should be emphasized that the formation of persistent disturbances of the function of the placenta is preceded by a violation of hemocirculation in the fetoplacental-fetal vessels, primarily due to increased pressure and vascular resistance [3]. Any treatment of PI is exclusively pathogenic or symptomatic, and the potential of medication to the fetus is limited to the morphofunctional capacity of the placenta. Pharmacotherapy PI includes the following groups of drugs [4-6]:

- Drugs that promote the relaxation of the muscles of the uterus-tocolitics (blockers of calcium channels, B-adrenomimetics, spasmolytics, cyclooxygenase inhibitors);
- Drugs that improve microcirculation and rheological properties of blood (antiagregants, angioprotectors, anticoagulants);
- Drugs that increase the resistance of the brain and tissues of the fetus to hypoxia (antihypoxants, neuroprotectors).
- Drugs for correction of disorders in the system of exchange of nitrogen oxide, the so-called precursors of synthesis NO (L-arginine).

The controlling role in the pathogenetic cascade of disorders of the

functioning of the placental endothelium belongs to nitric oxide [7-12]. Neurological deficits in children born from pregnancy with PI, is due primarily to the activation of apoptotic death of neurocytes.

Currently there are practically no works about pharmacological correction of neuroapoptosis after PI. We propose a combination of the essential amino acid L-arginine with thiotriazolol as a promising drug for fetoplacental dysfunction treatment. The biological functions of L-arginine are substantiated by the fact that it is a precursor to the synthesis of nitric oxide NO. The effectiveness of L-arginine in the complicated course of pregnancy is established in several studies [7-12]. There are no ways for using neuroprotectors with antiapoptotic effect in complex therapy of neurodestruction after placental insufficiency [13-15].

Recently, drugs with pronounced nootropic and neuroprotective effects are interesting in the treatment of PI and its effects. For the first time, piracetam was used for this purpose (2 g of piracetam in the form of 200 ml of 5% glucose solution by intravenous infusion over 30 min). The use of antioxidants Mexidol and Thiotriazoline has brought some success in the treatment of placental insufficiency and its aftermath.

Considering the role of endothelial vessels in the development of PI, use of NO-precursor L-arginine is justified.

Thiotriazolol (3-methyl-1,2,4-triazolyl-5-thioacetate and morpholine) reduces the formation of ROS in mitochondria, prevents oxidative modification of protein structures of receptors, ion channels, enzymes, transcription factors. Thiotriazolol may enhance activity of superoxide dismutase [16].

Combined nootropic, neuroprotective, antioxidant, anti-ischemic and antiapoptotic drug Thioacetam (combination of piracetam and thiotriazoline 1:4) was synthesized and applied in 2003 in Ukraine.

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We have proposed a combination of thiotriazolone and arginine (1:4) (Argitril), which exhibits anti-ischemic, antioxidant and endothelioprotective properties.

Our work describes neuroprotective effects of Thiocetam and Piracetam derivatives, that have been injected a pregnant rats with PI [17]. Intention of our work is to determine the antiapoptotic influence of Argitril, Thiocetam and Piracetam on neurons of rat brain hippocampus in case of experimental PI.

Materials and Methods

The research was carried out on female white rats weighing 150-180 g. All experimental procedures were carried out in accordance with the "Regulations on the use of animals in biomedical research".

The placental insufficiency model is reproduced by occlusion of preplacental vascular connections. Created "family" (at the rate of 1 male to 3-4 females). Cytological examination of the contents of the vagina was performed later 6-8 hours after mating, pregnant rats were isolated. On the 15th-16th day (the period of completion of the placentation and full transition to the placental circulation) the preplacental vascular areas one of the horns of the uterus were partially (about 50%) ligated (experimental horn), on the other horn a similar procedure was not done (intact horn).

The animals were fixed to the machine in the back position and under ether anesthesia according to the rules of asepsis, the abdominal cavity was opened in the middle line. Both uterine horns with fetuses were carefully removed to the surgical wound. The number of fetuses in both horns was counted, the fetuses were examined through the wall of the uterus, the state and degree of their development (presence of blood circulation in the area of fetus position, small sizes of the fetus) were recorded.

After numerating and revising the fetuses, the preplacental vascular branches (about 50%) were partially ligated directly at the position of each fetus of one of the horns of the uterus. After ligation of the vessels, the horns of the uterus with the fetus were successively immersed in the abdominal cavity and the wound was sutured. The duration of the operation was 5-6 min. Immediately after the operation, the animals were untied from the machine and placed in a personal cage, where they had a normal life. They had full access to food and water.

Thiocetam water solution was introduced intragastrically by a metal explorer immediately after emergence from anesthesia (250mg/kg) for 7 days until the onset of labor. Argitril was introduced similarly (250 mg/kg). Piracetam - in a dose of 500 mg/kg. In each group there were 10 pregnant females. After the physiological delivery on the 21-22nd day of gestation, newborns were observed. Ten rat pups from each group were selected by random sampling.

Study of the brain homogenized solution was done on the 30th day of the research. Blood was quickly removed from the brain, was separated from the dura mater and the test pieces were placed in liquid nitrogen. Then homogenized in a 10-fold volume at 2°C containing (in mmol): sucrose - 250, Tris-HCl-buffer-20, EDTA -1 (pH 7.4) [17]. The mitochondrial fraction was separated by the differential centrifugation method using a Sigma 3-30 k centrifuge at a +4°C.

For purification of the mitochondrial fraction from large cellular fragments, centrifugation was preliminarily carried out at 1000g for 7 minutes, and then the supernatant was recentrifuged at 17000g for 20 minutes. The supernatant was decanted and stored at -80°C.

For Enzyme Linked Immunosorbent Assays (ELISA) (ELISAKit (Cat. № HK 501-02) by HycultBiotech production) the brain tissue was

homogenized in the cold, in the salt isotonic solution (0,15M KCl) at +4°C [18]. Then cytosole and mitochondrial fractions are allocated at a temperature +4°C by method of differential centrifugation at refrigerate centrifuge Sigma 3-30 k.

Using ELISA, nitrotyrosine was determined in the mitochondrial and cytosolic fractions of the brain. Brain tissue are placed for a day in the Buens' solution for fixation before morphological and immunohistochemical research.

The study of the neurons morphology was done using CA-1 sections of the hippocampal zone and the sensorotom cortex, made by a rotary microtome, 5 microns thick. Hippocampal slices were dewaxed and stained to determine nucleic acids by gallocyanin-chromic alum by Einarson. Morphometric studies were performed on an Axioskop microscope (Zeiss, Germany), magnification x40.

The image of hippocampus neurons obtained with a microscope, was introduced into a computer-aided hardware digital system VIDAS (VIDAS-386 (Kontron Elektronik, Germany) using a highly sensitive video camera COHU-4922. This system developed by the professor of the Department of Pathophysiology, MD, A.V. Abramov. Image analysis was made in semiautomatic mode [18].

Neurons with karyopincosis or cytolysis were considered to be degenerating. The density of location of surviving and degenerating neurons, the number of intact neurons to dying (neurodegeneration index) and the density of surviving neurons using the drug to the density of intact neurons in the control group (survival improvement index) were measured by program.

Singly evaluated the relative activity index of microglia, because part of the dead neurons at the time of the histological study was already phagocytized by microglial cells [19,20]. This index is equal to the quotient of dividing the difference in the density of surviving neurons by the difference in density of degenerating neurons. The value of the neurodegeneration index of less than one testified to the predominance of the number of dying neurons over the surviving, the index of improvement in the survival and activity of microglia more than one indicated a positive effect of the pharmacological drug, less than one showed a negative effect. The functional state of the surviving neurons was judged on the basis of changes in the area of the nuclei and nucleoli of neurons, the content of nucleic acids in them, the nuclear-cytoplasmic ratio and the number of polynucleolar cells.

For histoimmunochemical studies, the brain of animals was moved to Buen's clamp per 18 hours. After the standard histological wiring, the tissue was placed in a paraplast. 15-micron sections of the hypothalamus were made on the rotary microtome, which were deparaffined according to the standard procedure. Immunofluorescence method detected the expression of c-fos and All-2 in the CA-1 hippocampal region.

Initially, the primary antibodies were applied to the c-fos and All-2 (Sigma Chemical, USA) antibodies and incubated at +40°C for 24 hours. After incubation, the slices were washed three times with 0.1 M phosphate buffer. Subsequently, secondary antibodies (fluorescence conjugated goat IgG) (Sigma Chemical, USA) were applied to the specimens and incubated at room temperature for 60 min.

After incubation, the slices were washed with 0.1 M phosphate buffer. The Axioskop fluorescence microscope (Zeiss, Germany) was investigated Fos-immunopolitical neurons and bcl-2-immunopolitical neurons. Image Fos-immunosuppressive neurons and bcl-2 immunopolitical CA-1 neurons of the hippocampal region obtained on a microscope using a high-sensitivity video camera COHU-4922

(COCHU Inc., USA) were injected into the computer software and hardware system of the digital image analysis VIDAS.

The concentration in the cytoplasmic or mitochondrial organ fractions (heart, brain) of bcl-2 was determined by Western-blot analysis. Proteins were separated in 10% polyacrylamide gel (PAAG). The separation of protein fractions was carried out by electrophoresis at a voltage of 100 V (to compact the gel), when the samples reached the boundary between the gels - at a voltage of 200 V, until the samples reached the end of the gel.

Proteins from the gel were transferred to a nitrocellulose membrane at a voltage of 100 V and a current of 0.35 A for 1 hour. After transfer, the membrane was placed in blocking buffer containing 1% solution of bovine serum albumin (SIGMA, USA, cat. No. A2153) for 20 h. The membrane washed on a shaker for 5 minutes in a solution of 0.1 M phosphate buffer was placed in a solution of primary antibodies against bcl-2 (1: 500) and incubated for 2 hours at room temperature. Washed on a shaker 4 times for 5 minutes in 0.1 M phosphate buffer. The membrane was placed in a solution of secondary antibodies (1: 1000) (biotinylated anti-mouse IgG, SIGMA, USA, cat. No. 051M4885), incubated for 2 h. Washed on a shaker 4 times for 5 minutes in a solution of 0.1 M phosphate buffer. The membrane was placed in a solution of ExtrAvidin-peroxidase (SIGMA, USA, cat. No051M4885) in a 1% solution of bovine serum albumin (1:1000). Incubated for 1 hour and washed. For visualization, the membrane was treated with a solution of AEK: 1 tablet of 3-amino-9-ethylcarbazole (Sigma, USA, cat. No. a6926) dissolved in 2.5 ml of DMF containing 47.5 ml of 0.05M acetate buffer, pH 5.0, 25 µL 30% H₂O₂.

The membrane was kept in the substrate during 5-10 min. The precipitate characterizes the antigen-antibody complex in the blot. Wash the membrane in distilled water several times. Dried strips between sheets of filter paper under a stream of cold air. The detection of bcl-2 was performed using densitometry in Adobe Photoshop.

The results of the research are processed using the program «STATISTICA for Windows 6.1» (StatSoft Inc., №AXXR712D833214FAN5), «SPSS 16.0», «Microsoft Office Excel 2003».

The normal distribution was estimated by the Shapiro-Wilk criterion. The significance of differences between the mean values was determined by Student's criterion with a normal distribution.

The U Mann-Whitney criterion was used in the case of a non-normal distribution or analysis of ordinal variables. For the comparison of independent variables in more than two samples, analysis of variance was used with a normal distribution or the Kruskal-Wallis test for a distribution other than normal. For all types of analysis, differences of $p < 0.05$ (95%) were considered statistically significant.

Results

Modeling of PI led to increase of postnatal death. So, in the intact group the number of newborns per 1 female was 9.7 ± 1.25 , in the group with PI it was 53.6% less (4.5 ± 0.7). PI led to an increase of rat pups mortality. The number of rat pups per 1 female with placental insufficiency was 3.5 ± 0.52 against 9.1 ± 1.0 in the control group (Table 1).

Drug therapy with Thiocetam (Thiotriazolone+Piracetam) and Argitril (L-arginine+Piracetam) resulted in a decrease in postnatal death. Thus, in the group where pregnant rats with PI received thiocetam, the number of live newborn rats per female was 46.7% more and 77.1% more the number of live rats per 1 female for 30 days of life than in the group of untreated animals for the same periods.

Argitril injection to females with PI resulted in a decrease in postnatal death by 73.3% and 105.7%, respectively. Our research determined that in the newborn rats brain born to mothers with PI was increasing of NO and inducing nitrosine stress (Table 2). NO and peroxynitrite plays not last role in induction of apoptosis. [9,10,15,16,21,22].

Discussion

Experimental therapy of animals with PI, lead to a decrease in the level of nitrotyrosine in both the mitochondria and the cytosol of the rat brain. According to Table 2, the greatest depression of the marker of nitrosine stress registered in the groups receiving Argitril and Thiocetam vs. a group of untreated animals and with the group, which provided only piracetam. The data obtained are consistent with our previous work, which demonstrated high antioxidant activity of Thiocetam and Argitril [14]. Argitril and thiocetam may provide the normal development of the cognitive function of the central nervous system [22]. Decreasing of nitrosine stress due to therapy and reduction of peroxynitrite, perhaps, promotes to oppression of neuroapoptosis [23].

Histoimmunochemical studies have identified that density of bcl-2-positive neurons in the CA-1 zone of the hippocampus was lower (88%)

Experimental groups	Number of live newborn rats per 1 female	The number of live rat pups on 1 female for 30 days of life
Rats, born in mother with normal pregnancy (n=10)	9.7 ± 1.25	9.1 ± 1.0
Rats, born in mother with PI (control) (n=10)	4.5 ± 0.7	3.5 ± 0.52
Rats, born in mother with PI and treated by Thiocetam(n=10)	$6.6 \pm 0.84^*$	$6.2 \pm 0.63^*\#$
Rats, born in mother with PI and treated by Argitril (n=10)	$7.8 \pm 0.63^*\#$	$7.2 \pm 0.63^*\#$
Rats, born in mother with PI and treated by Piracetam(n=10)	5.00 ± 0.8	4.5 ± 0.7

*Significant differences ($P < 0.05$) from the control group; #Significant differences ($P < 0.05$) from the group of piracetam.

Table 1: The effect of the studied combinations (Argitril, Thiocetam, Piracetam) on the survival of rat pups in mothers with PI.

Experimental groups	Concentration of Nitrotyrosine in cytosole fraction of the brain, nml/g protein	Concentration of Nitrotyrosine in mitochondrial fraction of the brain, nml/g protein
Rats, born in mother with normal pregnancy (n=10)	31.1 ± 1.1	10.7 ± 0.4
Rats, born in mother with PI (control) (n=10)	88.2 ± 4.6	38.0 ± 1.7
Rats, born in mother with PI and treated by Argitril (n=10)	$51.3 \pm 1.2^*\#$	$17.0 \pm 1.1^*\#$
Rats, born in mother with PI and treated by Thiocetam(n=10)	$61.7 \pm 2.0^*\#$	$22.1 \pm 1.5^*\#$
Rats, born in mother with PI and treated by Piracetam(n=10)	82.0 ± 2.2	30.8 ± 1.4

* $p \leq 0.05$ vs. vehicle-treated controls; # $p \leq 0.05$ vs. vehicle-treated piracetam.

Table 2: The effect of the studied drugs (Argitril, Thiocetam, Piracetam) on the content of nitrotyrosine in the brain of rat pups with PI.

in animals which have survived after PI at 25 days of life, than in intact rats group (Table 3). Treatment of PI by Argitril and Thiocetam, from 1 to 25 day of life increased the density of the bcl-2-positive neurons in the CA-1 zone of the hippocampus by 104, 6% and 67,6% respectively vs. the control group of animals. Low expression of antiapoptotic protein bcl-2 was determined by the method of immunoblotting in intact group. Morphometric studies revealed the appearance of apoptosis and destructive modified neurons in the CA-1 zone of the hippocampus animals with PI. Treatment by Argitril and Thiocetam during pregnancy of animals with PI decreased of apoptotic and destructive modified neurons CA-1 zone hippocampus by 87% and 77%, respectively vs. to the control group and the group of piracetam (Table 4).

Experimental group	Number of the bcl-2-positive neurons in 1 mm ²
Rats, born in mother with normal pregnancy (n=10)	211.7 ± 16.7
Rats, born in mother with PI (control) (n=10)	107.1 ± 7.5
Rats, born in mother with PI and treated by Argitril (n=10)	222.7 ± 11.6* [#]
Rats, born in mother with PI and treated by Thiocetam(n=10)	182.3 ± 9.1* ^{#1}
Rats, born in mother with PI and treated by Piracetam(n=10)	121.0 ± 10.3* ^{#1}

*p ≤ 0.05 vs. vehicle-treated controls; #p ≤ 0.05 vs vehicle-treated piracetam. 1-p ≤ 0.05 vs. vehicle-treated thiocetam

Table 3: The effect of drug combinations (Argitril, Thiocetam, Piracetam) on the density of the bcl-2-positive neurons of CA-1 zone hippocampus in rats born to mothers with PI.

The effect of drug combinations (Argitril, Thiocetam, Piracetam) on the number of apoptotic modified neurons of CA-1 zone hippocampus in rats born to mothers with PI. Introduction of argitril and thiocetam from 1 to 25th day of animals life treated with PI and argitril, significantly increasing the density of bcl-2-positive neurons in the CA-1 zone of the hippocampus higher vs. the control group and the group of piracetam. We established that the treatment by argitril and thiocetam increased the concentration of bcl-2 in the brain tissues of rats. In the group of argitril and thiocetam was happened antiapoptotic activation of neurons, in connection with the activation of bcl-2 (Table 5).

The pharmacological effect of Argitril is due to the positive effect on the synthesis, transport and bioavailability of NO and the physiological functions of this molecular messenger [18,21-23]. NO is an unstable, short-lived radical and mechanisms for its stabilization and subsequent transportation are provided such as the formation of stable S-nitrosol complexes with thio-containing low molecular weight compounds. In terms of deficiency of thiol compounds after placental insufficiency, NO transport is disturbed, since it is attacked by such ROS as superoxide radical and hydroxyl radical with transformation into a cytotoxic product - peroxynitrite. Argitril increases the level of reduced thiol groups, including activates glutathione reductase and direct reduction of the oxidized thiol group (due to the presence in the composition of thiotriazolone) (Figure 1).

In addition, the drug, due to its antioxidant properties, prevents

Animal group	Density of of apoptotic modified cells in 1 mm ²	Percent of apoptotic modified cells
Rats, born in mother with normal pregnancy (n=10)	3.05 ± 0.07	1.02 ± 0.05
Rats, born in mother with PI (control) (n=10)	13.11 ± 0.01	7.7 ± 0.05
Rats, born in mother with PI and treated by Argitril (n=10)	3.10 ± 0.03* ^{#1}	1.15 ± 0.02* [#]
Rats, born in mother with PI and treated by Thiocetam(n=10)	3.95 ± 0.03* [#]	1.77 ± 0.02* ^{#1}
Rats, born in mother with PI and treated by Piracetam(n=10)	10.44 ± 0.03*	1.77 0.06*

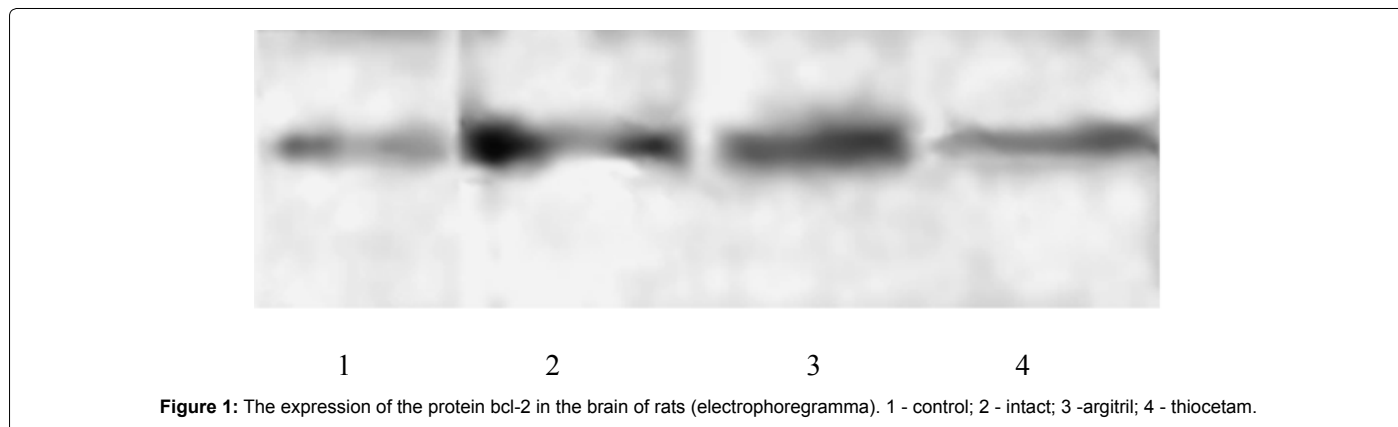
Note: *-p ≤ 0.05 vs vehicle-treated controls; #- p ≤ 0.05 vs vehicle-treated piracetam. 1-p ≤ 0.05 vs vehicle-treated thiocetam

Table 4: The effect of drug combinations (Argitril, Thiocetam, Piracetam) on the number of apoptotic modified neurons of CA-1 zone hippocampus in rats born to mothers with PI.

Experimental group	General proteins, g	Square, mm ²	Optical concentration, conventional units	Optical grade, conventional units
Rats, born in mother with normal pregnancy (n=10)	4.8 ± 0.02	58.27 ± 1.1	0.17 ± 0.001	6.81 ± 0.21
Rats, born in mother with PI (control) (n=10)	4.9 ± 0.01	57.32 ± 1.2	0.05 ± 0.01	0.89 ± 0.07
Rats, born in mother with PI and treated by Argitril (n=10)	4.8 ± 0.01	60.52 ± 1.0	0.19 ± 0.002* [#]	7.88 ± 0.10* ^{#1}
Rats, born in mother with PI and treated by Thiocetam(n=10)	4.9 ± 0.01	59.77 ± 1.2	0.15 ± 0.002* [#]	5.77 ± 0.11* [#]
Rats, born in mother with PI and treated by Piracetam(n=10)	4.8 ± 0.01	57.32 ± 1.3	0.06 ± 0.01*	0.98 ± 0.07*

Note: *- p ≤ 0.05 vs vehicle-treated controls; #- p ≤ 0.05 vs vehicle-treated piracetam; 1- p ≤ 0.05 vs vehicle-treated thiocetam

Table 5: The expression of the bcl-2 in the brain in rats born to mothers with PI.



the oxidative modification of NO by oxygen radicals. Argitril is able to act as a NO transport molecule, forming nitrosothiols [23,24]. Argitril also has a direct stimulating effect on NO synthase activity and NO production. Therefore, Argitril has unique properties to have a protective effect on the synthesis and transport of NO, it's bioavailability, which underlies the mechanism of such properties as fetoprotective and neuroprotective in relation to posterity. Argitril exhibits pronounced neuroprotective and antiapoptotic properties by reducing the destructive effect of free radicals on NO and reduces the formation of peroxynitrite, thereby inhibiting the NO-dependent pathway of apoptosis.

In addition, Argitril has an anti-apoptotic effect due to the regulation of ROS / SH- dependent expression mechanisms of nuclear transcription factors, bcl-2 antiapoptotic proteins. The neuroapoptotic effect of thiotriazolol based primarily in NO-dependent mechanisms. Argitril, due to the additional component of thiotriazoline, will have an advantage over piracetam. The mechanism of action of argitril, in our opinion, can be summarized as follows: an increase in the expression of the AP-1 transcription factor, the expression of the bcl-2 protein, the synthesis of superoxide dismutase. This is of great importance for the survival of neurons in hypoxic states.

Conclusion

In addition to this, argitril in accordance with our previous studies affects the activity of NO-synthase, increasing its synthesis. The result of this is the ability to modulate hypoxic-dependent nitrotyrosine stress. The consequence of all this is a decrease in the number of apoptosis in neurocytes [24]. In addition, according to some data, which coincide with our previous research, argitril modulates the activity of the mitochondrial synthase, thus limiting the intensity of nitrosine stress, as a result of which also inhibits neuroapoptosis. The results of this study may be interesting for further clinical study and use in the complex treatment of placental insufficiency.

Conflict of Interest

The protocols of experimental studies and their results were approved by the decision of the Bioethic Commission of ZSMU (protocol No. 33 dated October 26, 2017).

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