Available online at www.elixirpublishers.com (Elixir International Journal)

Pharmacy

Elixir Pharmacy 65A (2013) 20005-20008

ISSN: 2229-712X

Reduction of apoptotic death of neurons CA-1 zone of hippocampus of rats in the condition of prenatal chronic alcoholisation by cerebrocurin and tiocetam

Belenichev Igor Fedorovich, Sokolik Elena Petrovna and Egorov Alexander Nikolaevich

Department of Pharmacology, Zaporozhye state Medical University, Mayakovskiy str., 26, 69035, Zaporizhzhya, Ukraine.

ARTICLE INFO

Article history:

Received: 22 April 2013;

Received in revised form:

2 December 2013;

Accepted: 12 December 2013;

Keywords

Prenatal alcoholism,

Cerebrocurin,

Tiocetam,

CA-1 zone of hippocampus,

Apoptosis.

ABSTRACT

Our research found that prenatal alcoholism leads to increase of NO induction and nitrosine stress in the brain of newborn rats, evidenced by the increasing of nitrotyrosine in citosole and mitochondria. By adjusting the ratio of mitochondrial/cytosole concentrations of NO and reactive oxygen forms, cerebrocurin and tiocetam limited the effect of these compounds on the activation or deprivation of the processes of gene expression, transcription and translation in neuronal cells of brain of animals that survived the prenatal alcoholism and, thus, may provide the normal development of the cognitive functions of central nervous system. And increased expression of the protein bcl-2 in the group of animals receiving cerebrocurin and tiocetam, testifies to the activation of antiapoptosis protection of damaged neurons.

Introduction

There are a number of diseases in which apoptosis has a crucial role in the implementation of the mechanism of development of the pathology [1]. Violation of the oxidative function of mitochondria plays an important role in the pathogenesis of many neurodegenerative diseases, the result of this is reduction of energy equivalents in the cells and consequent reduction of the development of glutathione, increasing in the level of reactive oxygen species (ROS) and NO, that leads to the further development of apoptosis [2, 3]. At the present time neuroapoptosis is considered as one of the leading causes of cognitive frustration on the background of chronic alcoholism. In this case there are excessive generation of ROS and a significant deficiency of antioxidants in the organism [4].

It should be noted that increasing of the intracellular level of ROS may be caused by stimulation of the receptors of various types of cells of tumor necrosis factor-α (TNF-α), violation of the compensatory mitochondrial-cytosole shuttles of energy and metabolism of ethanol [5]. Currently there are practically no works about pharmacological correction of neuroapoptosis after prenatal chronic alcohol intoxication [6]. There are no ways for using neuroprotectors with antiapoptic effect in complex therapy of prenatal chronic consumption of alcohol. Our works describ neuroprotective effects of cerebrocurin, tiocetam and pyracetam for alcohol encephalopathy [7]. Based on the above, the purpose of this work is to estimate the antiapoptic actions of cerebrocurin, tiocetam and pyracetam in the condition of prenatal alcohol intoxication.

Materials and methods of research

The experiments were performed on female white rats weighing 150-180 g, obtained from the nursery of the state institution «Institute of Pharmacology and Toxicology Medical Sciences Academy of Ukraine». All experimental procedures were carried out in accordance with the "Regulations on the use of animals in biomedical research". Rats from 5-th to 20-th day of pregnancy received ethanol in the dose of 6-8 g/kg/day, control rats – isocaloric sugar solution. Seed of rats with chronic alcoholism received immediately after the birth during 25 days by intraperitoneal injection tiocetam (125 mg/kg), piracetam (125 mg/kg) and cerebrocurin (0.05 ml/kg), control group received saline solution. In each group were 20 newborns. Biochemical studies of the brain were held on the 26th day of the experiment, these animals were decapitated under thiopental anesthesia (30 mg/kg). The allocation of mitochondrial and cytoplasmic fractions of the brain, conducted by the method of differential centrifugation of McIlvaine and Rodnight.

For immunoenzyme research the tissue of the brain was homogenizated in the cold, in the salt isotonic environment (0,15M KCl) at a temperature +4°C with glass homogenizer, in the ratio of tissue:salt solution 1:20 [8]. Then cytosole and mitochondrial fractions are allocated at a temperature +4°C by method of differential centrifugation at refrigerate centrifuge Sigma 3-30k (Germany). It was determined nitrotyrosine in mitochondrial and cytosol fractions of the brain with ELISA-set of NITROTYROSINE by HBT-production, which is a solid phase of enzyme-binding immunosorbent set, operating on the principle of «sandwich». For morphological and histoimmunochemical research of brain tissue of experimental animals are placed for a day in the Buens` solution for fixation and after standard histological wiring the tissue is placed in paraplast X-TRA, after which rotating microtome made the sections of the studing areas of the brain with a thickness of 5 microns (for morphometric studies) and 15 microns (for hystoimmunochemical research) [9].

For the study of morphology of neurons and gliacytes the sections were stained for determination of nucleic acid by gallociannine-chrome alum by Ainarson.

Tele:

E-mail addresses: sokolikep@gmail.com

We were defined the following indicators:
- Density of apoptotic and destructive modified neurons as the number of cells in the 1мм^2 square of the cut;
- Cellular composition of the CA-1 zone of hippocampus in percent [10].

For histoimmunochemical study we used a method of indirect immunofluorescence. To determine the intensity of expression of antiapoptotic protein bcl-2 histological sections were isolated from paraplast and rehydratated three times at 5 minute and washed by phosphate buffer (pH=7.4) and within 30 minutes incubated with 2H hydrochloric acid (T=37^o C). Then histological sections were incubated within 24 hours in the damp cell (T=4-6^{0}C) with the primary polyclonal antibodies of rabbits Ig C (1:500) of bcl-2 developed by Santa Cruz Biotechnology, Inc. (USA). After incubation the slices are washed four times in 5 minutes by phosphate buffer (pH=7.4 per). Then within 1 hour (T=37^o C) incubated with secondary antibody goats to track mouse Ig G conjugates with fluorescence dye (FITC) of the company Sigma-Aldrich (cat no. F2266). After the final four times washing by phosphate buffer (pH=7.4 per) sections contained in the mixture of glycerin-phosphate buffer (9:1). At fluorescent microscope Axioskop (Ziess, Germany) defined the intensity of the expression bcl-2 by the density of the bcl-2-positive cells in the sections with the camcorder COHU-4922 (USA) and injected into the system of digital image analysis VIDAS-386 (Kontron Elektronic, Germany).

Also the quantity of bcl-2 proteins were determined by the method of immunoblotting. With this purpose the neurons of the cerebral cortex were allocated in two stages [11]. At the first stage the brain tissue disintegrated with the purpose of reception of cell suspension, the second was the differential ultracentrifugation. For the preparation of protein samples, cells were collected and separated from the substrate mixture by solutions of trypsin and versen (1:1), washed three times in 10 ml of cold PBS, centrifugated at 200 g for 5 min. To the cellular draft added 100 mkl of lytic buffer, consisting of 20 mm Tris-HCl, pH 7.5, 150 mm NaCl, 0.5% Triton X-100, 2 mm EDTA and 1 mm PMSF (Sigma production, USA). Extracts centrifugated at 8000 g for 10 min, selected supernatant and measure the concentration of total protein by the method of Bradford (1976). Electrophoretic separation of proteins were carried out by the method of Laemmli (1970). After transfer of proteins from the gel to the nitrocellulose membrane there were incubated for 1 h with monoclonal antibodies to the bcl-2, and with secondary antibody against immunoglobulin G (Ig G) of the mouse, labeled with peroxidase (Sigma, USA).

The results are presented in the form of a sample mean value or standard error of the mean values. The reliability of differences between the experimental groups were conducted with nonparametric U-test Mann-Whitney. Reliable considered differences with a significance level of more than 95% ($p<0.05$). The research results are processed using the statistical package license of the program «STATISTICA for Windows 6.1» (StatSoft Inc., No AXX R712D833214SAN5), «SPSS 16.0», «Microsoft Excel 2003».

Results and their discussion

Cerebrocurin – neuropeptide of new generation, received from embryos of large horned livestock. Cerebrocurin contains free amino acids, neuropeptides and low-molecular products of controllable proteolysis low-molecular fibers and peptides of embryos of large horned livestock.

As it known, the embryo at an early stage of ontogenesis contains the greatest concentration of regulative neuropeptides which at appropriating technological processing lay the basis of Cerebrocurin. It is not excluded, that in initial suspension of a preparation can get and neuroblast stam cells.

Regulative neuropeptides, making the basis of preparation, assist remielinisation, gliale proliferation and regenerations new neurons.

The procedure of preparing of Cerebrocurin consist of some stage. Tissue of a brain take from an embryo of animal, and homogenization weight dilute with a physiological solution then maintain it before completion of processes extraction, and to the solution received after removal of the formed deposit, add preservative in quantity not less than 0,5 %.Make sterilization of a solution by filtering and maintain it before completion of formation lipid layer, and after its branch remained solution maintain before the termination of processes of aggregation at temperature, not exceeding physiological. Then after branch of the formed particles solution subject to interoperability with immobilize proteolysis enzyme, and mode of interoperability establish, proceeding from control test of received means, and the received solution maintain within 30 day at temperature not above 10^{o}C.

Our investigations established that the prenatal alcohol intoxication (PA) leads to increase of NO and inducted nitrosine stress in the brain of a newborn rats, evidenced by the increase of nitrotyrosine in cytosole and mitochondria by 63% and 73% respectively in the control group in comparison with the intact group (table 1). Active derivatives NO are powerful inductors of neuroapoptosis in the opinion of many authors and our data [12]. NO and peroxynitrite plays not last role in posttranslation modification of caspase, participating in the S-nitrosation. The radical group NO was transported on cysteine of active center of the caspase with the formation of the group R-S-NO [13].

Table 1. The impact of investigational medications on the content of nitrotyrosine in the brain of animals with prenatal chronic alcoholisation

Experimental group	Nitrotyrosine in cytosole fraction of the brain, nml/g protein	Nitrotyrosine in mitochondrial fraction of the brain, nml/g protein
Intact	32.1±1.5	10.0±0.5
Control (PA)	87.2±4.8	37.2±1.8
PA+cerebrocurin	61.4±1.3*#	21.1±1.2*#
PA+tiocetam	41.1±2.1*#	16.0±1.4*#
PA+piracetam	85.0±3.5	30.7±1.3

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

Cerebricurin— neuropeptide of new generation, received from embryos of large horned livestock. Cerebricurin® contains free amino acids, neuropeptides and low-molecular products of controllable proteolysis, low-molecular fibers and peptides of embryos of large horned livestock. As it is known, the embryo at an early stage of ontogenesis contains the greatest concentration of regulative neuropeptides, which at appropriating technological processing lay the basis of Cerebricurin. It is not excluded, that in initial suspension of the preparation we can get neuroblast stem cells. Regulative neuropeptides, making the basis of preparation, assist remyelination, glial proliferation and regenerations of new neurons. The procedure of preparing Cerebricurin consist of some stages. Tissue of a brain taken from the embryo of an animal, then homogenization diluted with physiological solution, then maintain it before the extraction processes is completed. The solution is collected after removal of the formed deposit, and preservative is added in quantity not less than 0.5 %. Sterilize the solution by filtering and maintain it

before the completion of formation of lipid layer and after its branch remained solution maintain before the termination of processes of aggregation at temperature, not exceeding physiological. Then, after the branching of the formed particles in the solution are subjected to interoperability, with immobilizing proteolysis enzyme, the mode of interoperability is established, proceeding from control test of received means, and the received solution is maintained within 30 day at a temperature, below 10^{o}C.

Conducted experimental therapy from 1 to 25 days of life of animals that have suffered PA, lead to the decrease in the level of nitrotyrosine as in the mitochondria so in cytosole of the brain of rats. As can be seen from table 1, the greatest depression of the marker of nitrosine stress registered in the groups receiving tiocetam (53% - cytosole and 57% - mitochondrial fractions) and cerebrocurin (29% - cytosole and 43% - mitochondrial fraction) in comparison with a group of untreated animals and with the group, which provided piracetam. The data obtained are consistent with our previous work, which demonstrated high antioxidant activity of tiocetam and cerebrocurin [14]. By adjusting the ratio of mitochondrial/cytosole concentrations of NO and ROS cerebrocurin and tiocetam limit the effect of these compounds on the activation or deprivation of the processes of gene expression, transcription and translation in neuronal cells of the brain of animals that survived the PA and, thus, may provide the normal development of the cognitive function of the central nervous system [15]. Braking reactions of nitrosine stress after therapy and reduction of proapoptotic peroxynitrite ($ONOO^-$), apparently, contributes to inhibition of neuroapoptosis[16].

So, histoimmunochemical studies have shown that density of bcl-2-positive neurons in the CA-1 zone of the hippocampus was significantly lower (88%) in animals which have survived PA at 25 days of life, than in intact rats group (table. 2).

Table 2. The impact of investigational medications on the density of the bcl-2-positive neurons of CA-1 zone hippocampus in animals with prenatal chronic alcoholisation

Experimental group	Density of the bcl-2-positive neurons in 1 mm^2
Intact	211.8±17.0
Control (PA)	112.2±10.8
PA+cerebrocurin	235.7±21.7*#
PA+tiocetam	198.5±11.0*#
PA+piracetam	114.0±12.5

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

Introduction of cerebrocurin and tiocetam to animals, treated with PA, from 1 to 25 day of life had lead to a significant increase of the density of the bcl-2-positive neurons in the CA-1 zone of the hippocampus by 111% and 77% respectively compared with the control group of animals. In control group, against the background of alcoholism among us for the first time determined the method of immunoblotting low expression of antiapoptotic protein вс1-2 in animals treated with PA. It is known that bcl-2 is metalcontained protein, damption of free radicals, inhibits the development of apoptosis. Protein bcl-2 prevents cell death and functions as intracellular antioxidant. A recent analysis of the proteins of the family of gene bcl-2 revealed a complex network of reactions, regulating apoptosis. Within a family gene bcl-2 some of the participants could suppress apoptosis, and others call it [17]. Among the proteins encoded by the genes of this family, bcl-2 and bcl-x1 acted as repressor of cell death, while bax and alternative product of bcl-x caused the death of cells [18]. If bax prevailed over the bcl, it prevents the overwhelming impact of bcl-2 in apoptosis. Thus, the balance between the molecules of the bcl-2, bax bcl-xl/s can determine the fate of the neurons under the action of cytotoxic agents or stress. It is known that peroxynitrite lowers the level of bcl-2 expression. Morphometric studies have revealed emergence of apoptosis and destructive modified neurons in the CA-1 zone of the hippocampus animals underwent the PA.

Introduction of cerebrocurin and tiocetam from 1 to 25th day of animals life treated with PA, provided significant decrease of apoptotic and destructive modified neurons CA-1 zone hippocampus by 87% and 77%, respectively, compared to the control group and the group of animals receiving a course of piracetam (table. 3).

Table 3. The impact of investigational medications on the density of apoptotic/destructive modified neurons of CA-1 zone hippocampus in animals with prenatal chronic alcoholisation

Experimental group	Density of cells in 1 mm^2	Percentage of apoptotic modified cells
Intact	3.07 ± 0.08	1.07 ± 0.08
Control (PA)	11.23 ± 0.10	7.7 ± 0.31
PA+cerebrocurin	2.82 ± 0.10*#	1.0 ± 0.07*#
PA+tiocetam	3.67 ± 0.12*#	1.78 ± 0.12*#
PA+piracetam	9.58 ± 0.34	6.28 ± 0.31

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

Introduction of cerebrocurin and tiocetam from 1 to 25th day of animals life treated with PA, significantly increasing the density of bcl-2-positive neurons in the CA-1 zone of the hippocampus higher compared with the control group and the group that received the same pattern of piracetam. We also found that the course introduction cerebrocurin and tiocetam significantly increases the concentration of bcl-2 in the tissues of the brain of experimental animals. Increased expression of the protein bcl-2 in the group of animals receiving cerebrocurin and tiocetam, testifies to the activation antiapoptotic protection of damaged neurons (table. 4).

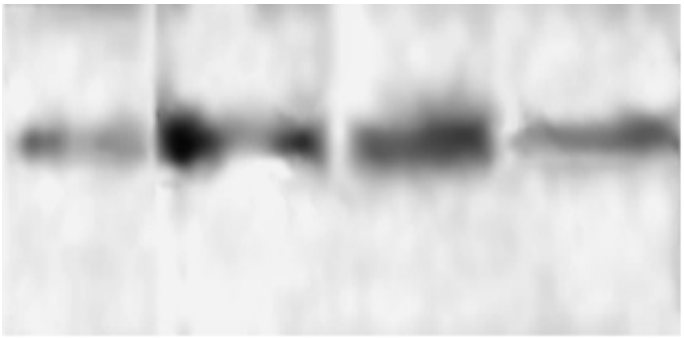
Table 4. The expression of the protein bcl-2 in the brain of animals with prenatal chronic alcoholisation

Experimental group	General proteins, g	Square, mm^2	Optical concentration, conventional units	Optical grade, conventional units
Intact	4.8±0.02	58.27±1.1	0.17±0.001	6.81±0.21
Control (PA)	4.9±0.01	57.32±1.2	0.05±0.01	0.89±0.07
PA+cerebrocurin	4.8±0.01	60.52±1.0	0.19±0.002*#	7.83±0.10*#+
PA+tiocetam	4.9±0.01	59.77±1.2	0.15±0.002*#	5.12±0.11*#
PA+piracetam	4.8±0.01	57.32±1.3	0.06±0.01*	0.97±0.07*

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

The expression of the protein вс1-2 under the influence of cerebrocurin and tiocetam suppresses nitrosine stress and apoptosis induced by action of ethyl alcohol. Cerebrocurin suppressed all manifestations of apoptosis (products of ROS, and fragmentation of the nucleus in the neurons of CA-1 zone of the hippocampus, decrease the number of apoptotic modified cells) on the background of hyperexpression of вс1-2 (photo) and by the strength of antiapoptotic actions reliably exceeded tiocetam and piracetam.

Antiapoptotic mechanism of neuroprotective actions of tiocetam due to its ability to form component (3-methyl-1,2,4-triasolil-5-thyoacetat), reduce early NO-initiation of apoptosis as scavenger of cytotoxic active derivatives NO [19].



1 2 3 4

Photograph. The expression of the protein bcl-2 in the brain of rats (electrophoregramma). 1 - control; 2 - intact; 3 - cerebrocurin; 4 - tiocetam.

In addition, tiocetam is able to regulate the synthesis of protein and Red/Oxi-dependent expression of the global transcription factors, which take part in the processes of memory and also increase the speed of the turnover of informational macromolecules, protecting their structures from oxidative modification, shows energotrope action, and due to this modulate the level of вс1-2 in the neurons [20].

However, seen from the obtained results, the domination of NO-dependent mechanisms neuroapoptotic actions does not provide to tiocetam high antiapoptotic activity as cerebrocurin. The fact that the mechanism of antiapoptotic actions of cerebrocurin, in our opinion, is connected with its ability to influence the genome of neurons in the extreme state, namely: increases the expression of the global transcription factor AP-1, increases the expression of antiapoptotic protein bcl-2, and also intensifies the synthesis of the main enzymes of antioxidant protection – Zn-Cu-SOD and Mn- SOD, the expression of glutathione-dependent enzymes. In addition, according to some data, which coincide with our previous research, cerebrocurin modulates the activity of the mitochondrial NO-synthase, thus limiting the intensity of nitrosine stress, as a result of which also inhibits neuroapoptosis[21]. Thus, in the result of the research we found that the classic nootropic therapy – piracetam does not suppress neuroapoptose, arising as a result of the PA, combined neurometabolic cerebroprotector tiocetam shows antiapoptotic effect due to the antioxidant mechanism, and the most pronounced antiapoptotic action has neurotrophic cerebroprotector cerebrocurin, increasing the expression of antiapoptotic proteins and slowing down the Red/Oxi-dependent mechanisms of neuroapoptosis. The obtained results are experimental rational for the clinical application of cerebrocurin and tiocetam in the complex phase-by-phase treatment of lesions of the central nervous system due to prenatal alcoholisation.

Reference

1. Kashihara N. Implication of apoptosis in progression of renal disease / N. Kashihara, H. Sugiyama, H. Makino // Contrib. Nephrol. – 2003. – Vol. 139:156-172.
2. Liaudet L. Biology of nitric oxide signaling / L. Liaudet, F.G. Soriano, C. Szabo // Crit. Care Med. – 2000. – Vol. 28(Suppl):N37-N52.
3. Bauer G. Reactive oxygen and nitrogen species: Efficient, selective, and interactive signals during intercellular induction of apoptosis / G. Bauer // Anticancer Res. – 2000. – Vol. 20:4115-4139.
4. Chertin B.The role of nitric oxide in reflux nephropathy / B. Chertin, U. Rolle, H. Farkas // Pediatr Surg Int. – 2002. – Vol. 18: 630-634.
5. Belenichev I.F., Kolesnik Y.M., Pavlov S.V., Abramov A.V., Buhtiyarova N.V. Mitochondrial dysfunction in cerebral pathology. Neuroprotection by Cerebrocurin® / / by international neurological journal. - 2008. - № 4 (20). - S. 23-29.
6. Belenichev I.F., Levitskyy E.L., Pavlov S.V. The role of early response gene c-fos in normal and neurodestruktiv toxic pathology. Features farmakocorection neuropeptid drugs / / Modern problem of toxicology. - 2008. - № 1. - S. 17-27.
7. Belenichev I. F., Chernykh V.A., Kolesnik M.U. Rational neuroprotection / / Donetsk: A.U. Zaslavsky publisher, 2009.- 262s.
8. Levytskyy E.L., Hubskyy Y.I. Freeradicale damaged of nuclear genetic apparate of the cell. / / Ukr. Biochim. Gourn. - 1994. -T. 66, № 4. -C. 18-30.
9. You H.X. Atomic force microscopy imaging of living cells: progress, problems and prospects / H.X. You, L. Yu // Methods Cell Sci. – 1999. – V. 21, № 1. – Pp. 1–17.
10. Sokolovskyy V.V. / / Questions of medical chemistry. - 1998. -T. 6, № 34-C. 2-11.
11. Dubnina E.E, Morozova M.G., Havrovskaya S.V., Kuzmich E,V., Leonova N.V./ / Biochemistry. -2002. -T. 67, № 3. -S.413-421.
12. Belenichev I.F., Pavlov S.V., Sokolik E.P., Buhtiyarova N.V., New treatment possibilities of alkohole disease. Cerebrocurin® application prospects. / / International neurology gournal. - 2008. - № 2 (20).
13. Zaidi A., Miachaels ML / / Free Rad. Biol. -1999. -V. 27. - P. 810-821.
14. Dean RT, Hunt JV, Grant AJ et al. / / Free Rad. Biol. Med. - 1991. -V. 11, № 12.-R.161-165.
15. Granger D.N. Nitric oxide as antiinflammatory agent / D.N. Granger, P. Kubes // Methods in Enzymology. – 1996. – V. 269. – P. 434 – 442.
16. Rice-Evans C.A. Laboratory techniques in biochemistry and molecular biology: techniques in free radical research / C.A. Rice-Evans, A.T. Diplock, M.C.R. Symons // Elsevier. – 1991. Elsevier Amsterdam-London-New York. Tokyo. – P. 291.
17. Yena L.M., Kuznetsova S.M., Kuznetsov V.N. and others. Material and experimental clinical trials of preparation 'Cerebrocurin® ®' / / Kyiv, 1997. - 115 pp.
18. Adams J. Vascular endothelial growth factor (VEGF) in breast cancer: comparison of plasma, serum, and tissue VEGF andmicrovessel density and effects of tamoxifen / J. Adams, P.J. Carder, S. Downey // Cancer Res. – 2000. – Vol. 60 (ll). – P. 2898-2905.
19. Ahmad S. Role of AKT1 in 17beta-estradiol- and insulin-like growth factor I (IGF-I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells / S. Ahmad, N. Singh, R.I. Glazer // Biochem Pharmacol. – 1999. – Vol. 58 (3). – P. 425-430.
20. Al-Moundhri M. Significance of p53, Bcl-2, and HER-2/neu protein expression in Omani Arab females with breast cancer / M. Al-Moundhri, V. Nirmala, K. Al-Mawaly // Pathol Oncol Res. – 2003. – Vol. 9 (4). – P. 226-231.
21. Altiok S. Heregulin induces phosphorylation of BRCA1 through phosphatidylinositol 3-Kinase/AKT in breast cancer cells / S. Altiok, D. Batt, N. Altiok // J Biol Chem. – 1999. – Vol. 274 (45). – P. 32274-32278.