PHARMACOLOGICAL MODULATION OF APOPTOSIS SIGNALING IN NEURONS OF CA1-ZONE OF HIPPOCAMPUS OF RATS WITH CHRONIC ALCOHOL INTOXICATION.

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ABSTRACT

Patients with chronic alcohol intoxication have diffuse changes extended on all nervous system and local defeat (parenchymal disintegration, gliosis hems, haemorrhage). Research on new ways of pharmacocorrection of morphofunctional changes in neuro-glial structures of the brain and restoration of interneural interactions at modelling of 30-day chronic alcoholic intoxication in rats, has defined effective neuropeptide cerebroprotectors (cerebrolisin, cortexin and cerebrocurin). Also, results of experiment have shown that the most active preparation is cerebrocurin, so it is recommended for inclusion in the traditional scheme in treatment of chronic alcoholic intoxication.

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INTRODUCTION

Alcoholism is an urgent social and medical problem of our time, which requires the search for new ways of its solution. Alcohol intoxication, which lasts for years, lead to persistent morphological changes in various organs [1-2]. It was pointed out by different researchers that patients with chronic alcohol intoxication had diffuse changes, that are common throughout the nervous system and focal lesions (local parenymal destruction, gliosis scarring, bleeding, located in certain places, mainly under the ependima at the bottom of III ventricle in Silvias water supply); there are also changes in the peripheral nerves [3-4]. However, despite the progress achieved in the study of this issue, the problem remains urgent. Up to the present time the molecular mechanisms of neuronal death under the action of ethanol and its metabolites have not been understood. Study of intimate mechanisms of running processes (neuropoptosis or necrosis), will allow us approach the appointment of neuroprotection in the conditions of alcoholism more seriously. Taking into account the results we and other researchers obtained in the field of alcohol neurodestruction: it is expedient, to consider the application of neurotrophic cerebroprotectors (cerebrocurin, cortexin, cerebrolisin), which have a therapeutic effect on various pathologies of the central nervous system [5-6].

THE AIM OF THE RESEARCH

It is on the basis of experimental studies of molecular and morphological changes in the brain of rats subjected to abuse that the application and the assessment of neuroprotective actions of neuropeptides drugs (cerebrocurin, cortexin, cerebrolisin) is justified.

MATERIALS AND METHODS
In the experiments we used 50 white outbred rats (males) with body weight 180-220 g and age 4.5 months, which were contained in the vivarium with free access to food (standard granulated food) and water also with natural changing of day and night. The animals were 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" [7-8].

Chronic alcohol intoxication was achieved by daily intragastral introduction during the first 10 days – 15% solution of ethanol in doses of 4g/kg, next 10 days – 15% solution of ethanol in doses of 6g/kg and last10 days rats get 25% ethanol solution in doses of 3g/kg (R. Mirzoyan, 2001). During all phase of alcoholization we conducted experimental drug therapy and continued surveillance within all 30 days. The 50 rats used in these experiments were randomly divided into five groups (n=10) (four experimental groups and one control group):
Group 1 received ethanol and Cerebrocurin® within 30 days in dose 0.06 mg/kg,
Group 2 received ethanol and Cerebrolizin® within 30 days in doses 4 mg/kg,
Group 3 received ethanol and Cortexin® within 30 days in the dose 0.5 mg/kg,
Group 4 received ethanol within 30 days (control),
Group 5 - intact (instead of ethanol received saline solution) [9-10].

For the morphological studies the brain tissue of experimental animals was placed for a day in the Buen solution-fixator and after standard histological wiring, the tissue was kept in paraffin [11]. For the morphological study of neurons in the rotating microtome made slices CA-1zone of hippocampus with a thickness of 5 mm. Sections of the hippocampus deparafineted and stained for
determination of nucleic acids by gallicanin-chrome alum by Einarsone [12]. Morphometric investigations were carried out on the microscope Axioskop (Ziess, Germany), increasing ×40. The image of neurons in the area CA-1 of hippocampus was received on the microscope, with the help of highly sensitive camcorder COHU-4922 (COCHU Inc., United States of America) provided in a computer-hardware-software system of digital image analysis VIDAS. Image analysis was carried out in semi-automatic mode [13].

To determine the content of the bcl-2 protein, we allocated the neurons of the cerebral cortex in two stages. At the first stage, the brain tissue was disintegrated with the purpose of receptor cell suspension, the second was the differential ultracentrifugation. **By method of immunoblotting** we defined the concentration of proteins bcl-2. For the preparation of protein samples, cells were collected, separating them from the substrate mixture of solutions of trypsin and versen (1:1), washed three times in 10 ml of cold PBS, centrifugated at 200 g for 5 min. The cellular draft added 100 microliters 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 production (Sigma, USA). Extracts centrifugated at 8000 g for 10 min, we selected supernatant and measured in it, the concentration of total protein, by the method of Bradford (1976). Electrophoresis separation of proteins was carried out by the method of Laemmli (1970). After transferring proteins from the gel to the nitricellulose membrane, it’s incubated for 1 h with monoclonal antibodies to the bcl-2, and with secondary antibody against immunoglobulin G (IgG) of the mouse, labeled with peroxidase (Sigma, USA) [14-15].

**Statistical analyses** were performed with the use of "STATISTICA for Windows 6.1" (StatSoft Inc., № AXX R712D833214SANS5), and "SPSS 16.0", "Microsoft Excel 2003". When multiple comparisons were indicated, Dunnett’s test or the Student-Newman-Keuls test was applied. Differences were considered significant at the P<0.05 level. Results were presented as means±SD.

**RESULTS AND DISCUSSION**

**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 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 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 maintain within 30 day at a temperature, below 10°C.

**Cortexin**— a multicomponent preparation; its components are presented by L-amino acids, vitamins and mineral substances. Peptides of Cortexin® consist of some amino acids: aspartic acid (446 nm/mg), treonine (212 nm/mg), serine (268 nm/mg), glutamic acid (581 nm/mg), proline (187 nm/mg), glycine (298 nm/mg), alanine (346 nm/mg), valine (240nm/mg), isoleucine (356 nm/mg), tyrosine (109 nm/mg), phenylalanine (162 nm/mg), hystidin (116 nm/mg), lysine (253 nm/mg), arginine and others (202 nm/mg). On the fraction of aspartic acids it is necessary up to 12 %, and glutamic acids — about 15 % from the general content of amino acids in structure of peptides. Glycine, present at a preparation, at the same time carries out a role of the stabilizer. Into the structure of Cortexin®, water-soluble (thiamine — 0.08 mgk/10 of mg, riboflavin— 0.03 mgk/10 of mg, niacin — 0.05 mgk/10 of mg) and fat-soluble vitamins (retinol— 0.011 mgk/10 of mg, an alpha-tocopherol — 0.007 mgk/10 of mg) enter. At the preparation there are mineral substances (Cu — 0.2129 mgk/10 of mg, Fe — 2.26 mgk/10 of mg, Ca — 22.93 mgk/10 of mg, Mg — 8.5 mgk/10 of mg, K — 19.83 mgk/10 of mg, Na — 643.2 mgk/10 of mg, S — 152.65 mgk/10 of mg, P — 91.95 mgk/10 of mg, Zn — 4.73 mgk/10 of mg, Mb — 0.0203 mgk/10 of mg, Co — 0.0044 mgk/10 of mg, Mn — 0.0061 mgk/10 of mg, Se — 0.0745 mgk/10 of mg, Al — 0.3104 mgk/10 of mg, Li — 0.0340 mgk/10 of mg). Supposed that the positive effect of Cortexin® speaks not only for the action of polypeptides components, but also for neurochemical activity, macro-and microelements, as well as vitamins (A, E, B1 and PP).

**Cerebrolisin®** has been officially allocated an active fraction. It consist of the balanced and stable mixture of amino acids (85 %), biologically active neuropeptides (15 %) possessing total multifunctional action. However the structure of Cerebrolisin® is more complex. Early researches have shown that in cleared Cerebrolisin®, more than 100 oligopeptides and motives of protein with weight basically up to 5800 Da are present; these are numerous short combinations of amino acids and fragments peptides, received at trispinolisis proteome cortex of a pig’s brain. They represent the potential for metabolism of nervous cells in a trophic product. The important result of the research was tracking down in structure of Cerebrolisin®, vital for neurochemistry of oligopeptides in brain. It’s tripeptides are glutathione (Glu-Cys-Gly) and thyroliberin (Glu-His-Pro); as well as encephalin (Tyr-Gly-Gly-Phe) and collagen (Gly-Pro-Hyp). Earlier membrane fraction of lipids and possible effects of Cerebrolisin®, connected with the increase of neuron’s plasticity were discovered; which defends on the action of not only membrane fraction of peptides, but also on heterogeneous fraction of neurospecific lipids.

Modeling of chronic alcohol intoxication, led to persistent violations of histostructural CA-1 zone of the hippocampus and to the development of apoptosis. These changes are expressed in the reliable decrease of neuron’s density in the 30-day alcohol intoxication to 892.2±147.82 neuron/mm² in comparison with the intact animals, from which this date was 1389.8±275.65 neurons/mm² (fig.1).
important in subcellular distribution of Ca$^{2+}$ between the nucleus, mitochondria and endoplasmic reticulum [22-24]. A decrease in expression of anti-apoptosis protein Bcl-2 was determined in the control group with alcohol intoxication (table 1).

**Table 1 Expression of protein Bcl-2 in the brain of rats with chronic 30-day alcohol intoxication.**

<table>
<thead>
<tr>
<th>Group of animals (N=10)</th>
<th>Total protein, grams</th>
<th>Area, mm$^2$</th>
<th>Optical concentration, conventional units</th>
<th>Optical grade, conventional units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>4.7</td>
<td>61.43</td>
<td>0.14</td>
<td>6.02</td>
</tr>
<tr>
<td>Control</td>
<td>4.8</td>
<td>59.08</td>
<td>0.02</td>
<td>1.06</td>
</tr>
<tr>
<td>Cerebroisin</td>
<td>4.7</td>
<td>54.21</td>
<td>0.09*</td>
<td>4.35*</td>
</tr>
<tr>
<td>Kortexin</td>
<td>5.0</td>
<td>51.12</td>
<td>0.10*</td>
<td>4.81*</td>
</tr>
<tr>
<td>Cerebrocurin</td>
<td>4.9</td>
<td>49.94</td>
<td>0.12*</td>
<td>5.94*</td>
</tr>
</tbody>
</table>

*P<0.05 vs vehicle-treated controls

Overexpression of protein Bcl-2 in groups of animals receiving neuroprotective drugs was determined with activation of antiapoptosis protection of damaged neurons (fig. 2)

**Figure 2. Expression of the protein Bcl-2 in the brain of rats.**

1 - control group; 2 - intact group; 3 - cerebrocurin group.

Experimental therapy of animals with introduction of cerebrolisin, cortexin and cerebrocurin, demonstrated the effect of neuroprotective actions, by increasing the density of neurons by 23.28%, 34.43% and 44.87%, respectively, compared to the control group of animals (table 2).

**Table 2 The impact of cerebrolisin, cortexin and cerebrocurin on the density of neurons, area of neurons bodies, content of RNA in the zone of CA-1, in the hippocampus of rats with chronic alcoholic intoxication.**

<table>
<thead>
<tr>
<th>Group of animals (N=10)</th>
<th>Density of neurons (neuron/mm$^2$)</th>
<th>Area of neurons (mm$^2$)</th>
<th>Content of RNA (E$^{optical}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>1389.8±275.65</td>
<td>155.8±37.35</td>
<td>14.1±2.85</td>
</tr>
<tr>
<td>Control</td>
<td>992.2±147.82</td>
<td>106.8±23.72</td>
<td>9.8±1.78</td>
</tr>
<tr>
<td>Cerebroisin</td>
<td>1099.9±251.02*</td>
<td>119.2±21.10*</td>
<td>12.0±2.37*</td>
</tr>
</tbody>
</table>
Also neurotrophic drugs (cerebrolisin, cortexin and cerebrocubin) increased the area of neurons in the zone of CA-1 hippocampus of rats to acquire 11.61%, 28.75% and 40.54% respectively in relation to the control group and the content of the RNA to 22.96%, 31.63% 40/82% respectively in relation to the control group.

In the study of glial cells, we have recorded a reduction of the density of glial cells in the control group to 399.9±85.57 of neurons in the mm2, while the same indicator of the intact group was 445.9±101.98 of neurons in the mm2.

After the course of neuroprotective therapy we found positive effect of cerebrolisin, cortexin and cerebrocubin. It increased the density of glial cells by 4.6%, 7.25% and 9.63% respectively as compared to the control group (table 3).

Table 3 Impact of cerebrolisin, cortexin and cerebrocubin on the density of glial cells, the area of glial cells, contents of RNA in the zone of CA-1 hippocampus of rats with chronic alcoholic intoxication.

<table>
<thead>
<tr>
<th>Group of animals (N=10)</th>
<th>Density of glial cells (neuron/mm²)</th>
<th>Area of glial cells (m²)</th>
<th>Content of RNA (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>445.9±101.98</td>
<td>25.7±5.83</td>
<td>5.85±0.81</td>
</tr>
<tr>
<td>Cerebrolisin</td>
<td>399.9±85.57</td>
<td>19.1±4.10</td>
<td>4.04±0.77</td>
</tr>
<tr>
<td>Cortexin</td>
<td>438.3±93.15</td>
<td>21.9±5.55</td>
<td>4.49±0.81</td>
</tr>
<tr>
<td>Cerebrocubin</td>
<td>420.9±99.41</td>
<td>22.9±4.94</td>
<td>4.6±0.89*</td>
</tr>
<tr>
<td>Cerebrocubin</td>
<td>438.4±97.30</td>
<td>24.9±7.77</td>
<td>5.5±0.9*</td>
</tr>
</tbody>
</table>

*P<0.05 vs vehicle-treated controls

The decrease of glial cells area in the control group to 19.1±4.10mm² was noted, while in the intact group this indicator, amounted to 25.7±5.83mm². Cerebrolisin, cortexin and cerebrocubin increased this date to 14.66%, 19.90% and 30.37% respectively in relation to the control group. Also, these drugs had positive influence on the content of RNA in glial cells, raising this date to 11.24% (cerebrolisin), 22.83% (cortexin) and the 36.21% (cerebrocubin) in relation to the control group.

Density of apoptotic cells in the control group reached indicator 178.5±44.86 to 1 mm², while in the intact group density of apoptotic cells was 86.2±15.68 on 1 mm² (table 4).

Table 4 Density of apoptotic and destructive abnormal cells in the zone of CA-1 hippocampus in the brain of rats with chronic alcoholic intoxication.

<table>
<thead>
<tr>
<th>Group of animals (N=10)</th>
<th>Density of apoptotic cells in 1 mm²</th>
<th>Share of apoptotic cells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>86.2±15.68</td>
<td>4.9±0.82</td>
</tr>
<tr>
<td>Control</td>
<td>178.5±44.86</td>
<td>15.2±3.25</td>
</tr>
<tr>
<td>Cerebrolisin</td>
<td>139.4±26.32*</td>
<td>10.9±2.15*</td>
</tr>
<tr>
<td>Cortexin</td>
<td>127.4±19.92*</td>
<td>9.8±2.30*</td>
</tr>
<tr>
<td>Cerebrocubin</td>
<td>107.6±24.36*</td>
<td>6.0±1.11*</td>
</tr>
</tbody>
</table>

*P<0.05 vs vehicle-treated controls

In the group of cerebrocubin the density of apoptotic cells decreased by 21.90%, in the group of cortexin - by 28.63%, in the group of cerebrocubin - 39.72% as compared to the control group. Accordingly neurotrophic drugs reduced the share of apoptotic cells to 27.76% (cerebrolisin), 35.39 (cortexin) and the 60.26 % (cerebrocubin) in relation to the control group.

On the basis of the obtained results, there was a positive impact of neurotrophic cerebrocubin (cerebrolisin, cortexin and cerebrocubin) on the area, density and contents of RNA in neurons and glial cells. Preparations caused a pronounced gliosis and increase in the content of RNA in glial cells, which indicated the level of functional activity of cells, activation of genes and protein synthesis. Gliosis is a compensatory mechanism, which begins from damage of nervous tissue. It should be noted also that therapy by this drugs, has led to considerable reduction of apoptosis, proved by decreasing density and division of apoptotic cells. Based on the obtained results, it can be argued that the most effective agent was cerebrocubin, surely leading in all studied indicators. This is consistent with our previous research, which proved that cerebrocubin is able to enhance the compensatory activation of anaerobic glycolysis, reduces degree of oppression by oxidizing processes in the Krebs cycle and stabilizes the membrane of neurons. Also, cerebrocubin increased the expression of the protein Bcl-2, which can testify to protection, by its damaged cells from apoptosis. Proceeding from this, it is possible to recommend cerebrocubin for inclusion, in the traditional scheme of alcoholism treatment, as one of the most promising neuroprotector.

CONCLUSIONS

1. Formation of chronic alcohol intoxication in rats within 30 days in the control group decreased the density, area and content of RNA in the neurons and glial cells of the CA-1 zone of the hippocampus in the brain and increased the density and division of apoptotic cells.

2. Prevention treatment of alcohol abuse by cerebrolisin, cortexin and cerebrocubin had positive impact on the area, density and contents of RNA in neurons and glial cells and increased the expression of antiapoptosis protein Bcl-2.

3. Determined the most effective preparation – cerebrocubin, which significantly exceeded the above-mentioned drugs in all studied indicators and is recommended for inclusion in the traditional scheme of alcoholism treatment.

REFERENCES


8. Хабрієв Р.У., Рекомендації по експериментальному (доклінічному) вивченню нових фармакологічних речовин./ Р.У. Хабрієв. – Москва, 2005 р.


10. Корекція енергетичного метаболізму нейропептидами в умовах хронічної алкогольної інтоксикації/ Беленічев І.Ф., Соколик Е.П. // Патологія -2010, том 7, №2, стр.50-53.


