Neuroprotective and Anti-apoptotic Activity of the IL-1 Antagonist RAIL-gel in Rats after Ketamine Anesthesia

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ABSTRACT

Introduction: The choice of medicines for total intravenous anesthesia remains a relevant issue in practical anesthesiology. Ketamine is a well-known drug that has been widely used in the world, however its' effect on the CNS is debatable. It is reasonable to question the use of neuroprotective agents to protect against the negative effects of general anesthesia. Some studies have shown the neuroprotective activity of the RAIL. A new dosage form of RAIL - a gel for intranasal administration has been developed. This study was designed to evaluate the neuroprotective and anti-apoptotic activity of RAIL-gel in comparison with Citicoline and Piracetam during ketamine anesthesia

Methods: In this study, 50 white nonlinear rats were randomly assigned to 5 groups: intact, ketamine anesthesia group, ketamine anesthesia + Piracetam (500 mg/kg, intraperitoneally) group, ketamine anesthesia + Citicoline (500 mg/kg, intraperitoneally) group, ketamine anesthesia + RAIL-gel (1 mg / kg intranasally) group. Expression of c-fos in the CA1 zone of the hippocampus and concentration of bcl-2 protein in the cytoplasmic fraction of the brain were determined by indirect immunofluorescence and Western blot analysis respectively.

Results: Our research demonstrated the neurodegradative effect of ketamine anesthesia. The use of neuroprotective agents (Piracetam, Citicoline, RAIL-gel) in rats after general anesthesia led to a decrease in the neurodegradative effect of ketamine. The neuroprotective effect of RAIL-gel was significantly higher compared to reference drugs ($p < 0.05$).

Conclusion: The neuroprotective effect of RAIL-gel is an experimental justification for further study of IL-1β RAIL antagonist as a potential neuroprotective agent.

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Introduction

The choice of medicines for total intravenous anesthesia remains a relevant issue in practical anesthesiology for many years. Requirements for drugs that cause anesthesia are numerous and varied - rapid onset of anesthesia, high efficiency, controllability, safety and more, but in recent years, these criteria have been supplemented by the absence or minimal negative impact on the central nervous system (CNS)[1].

Ketamine is a well-known drug that has been widely used in the world since the 1970s. Its mechanism of action is associated with non-competitive N-methyl-D-aspartate receptors (NMDA receptor) antagonism, leading to functional disorganization of nonspecific midbrain and thalamic ligaments, causing dissociative anesthesia[2, 3]. Ketamine was developed as a safe alternative to phencyclidine, the neurotoxicity of which has been known and experimentally proven[4-10]. For many years, scientific debate about ketamine effect on the CNS is going on. Some authors in their articles report data on the neuroprotective properties of this drug, suggesting its use in order to reduce brain damage in acute CNS lesions[50], or discuss its neuroprotection at the cellular level[6]. Others have experimentally demonstrated that it causes neuroapoptosis and prolonged behavioral disorders in rodents of different age groups, including newborns[7,11]. The neurotoxic effect of ketamine is due to the expression of c-fos early responsive genes[8, 12]. Furthermore ketamine reduces the total antioxidant capacity and causes excessive formation of reactive oxygen species (ROS) and malondialdehyde[13], and apoptosis levels have been shown to correlate with the level of 3-methyladenine autophagy inhibitor[8].

NMDA receptors' hyperstimulation is known to cause excess Ca2+ influx, leading to free radical formation, activation of proteolytic processes and, ultimately, neuron necrosis. At the same time, moderate activation of NMDA receptors leads to oxidative stress and neuroapoptosis[14]. Therefore, ketamine, as a non-competitive antagonist of NMDA receptors, should not cause excitotoxicity, but rather cause a neuroprotective effect. However, it has been experimentally demonstrated that ketamine anesthesia causes pervasive developmental disorder in the form of anxiety and cognitive disorders associated with neuroapoptosis[9]. There is a strong evidence for dose-dependent apoptotic neurodegeneration caused by ketamine in the immature brain of mice[15]. The mechanism of neuroapoptosis is associated with the ketamine induced manifested expression of c-fos in the posterior cingulate gyrus and in the retrospinal cortex. Ketamine-induced expression is mediated not only through NMDA receptors but also through sigma-receptors[12]. Apoptosis is a programmed cell death with a complex pathogenesis. The regulation of apoptosis in the nervous system is carried out by numerous signaling systems. One way to implement this process is through the direct activation of early immediate response genes (c-jun, c-fos). C-fos protein acts as a regulator of transcription of a number of inducible genes and plays a significant role in cellular growth and differentiation processes and appears a recognized marker of neuronal cell activation[16]. One of the defense mechanisms is the production of anti-apoptotic modulators, one of which is the bcl-2 protein, which action is associated with the normalization of mitochondrial function. There is a mechanism of caspase1-dependent apoptosis of CA1 hippocampal neurons after ketamine anesthesia, associated with the exit of proapoptotic proteins from mitochondria. It is established that the active caspase-3 and -9 proteins, which are responsible for the release of cytochrome C and the mitochondrial translocation of p53, which is associated with mitochondrial apoptosis, are found to be significantly activated after a single administration of ketamine. Also, the introduction of ketamine increases the levels of pyroptosis-related proteins, including caspase-1 and -11, the NOD-like receptor family, the pyrin domain containing 3 (NLRP3), and IL-1β[17]. Anesthesia with ketamine or thiopental is also known to increase IL-1 and TNFα levels in the hippocampus 2 hours after anesthesia[18].

According to modern ideas, the nature of the immune response and the development of pathophysiological changes in neurodegradation, both ischemic and non-ischemic, depends on the predominant

activation of subpopulations of T-lymphocytes, their synthesis of various types of cytokines and the formation of a "cytokine cascade," namely, the ratio of pro-inflammatory and anti-inflammatory cytokines[19]. In case of brain tissue damage, activated microglia begin to produce pro-inflammatory cytokines, primarily IL-1β, which contributes to an increase in cerebral edema and increases the adherence to endothelium[20,21]. IL-1β can increase the expression of iNOS and stimulate the NO-mechanisms of neurodegradation. This induces neuroapoptosis, leads to delayed neuronal losses and a decrease in cognitive and mnestic functions of the CNS[9,17,19].

Many scientific papers and researches are devoted to the medico-social problem of cognitive impairment after anesthesia and surgery known as postoperative cognitive dysfunction (POCD). POCD is a cognitive disorder that develops in the postoperative period (may be delayed in time) and is associated with impaired higher cortical function, more commonly manifested as impairment of memory, speech, decreased attention spans, and more[22-24]. The most accurate, attention-grabbing study of the occurrence of POCD is the international randomized controlled trial of the ISPOCD1 study, published in 1998. According to the results of this study, cognitive dysfunction was diagnosed in 8% of patients 7 days after surgery and 9.9% 99 days after surgery[8], which indicates the prevalence of this problem in the world and the relevance of its study. The etiology and pathogenesis of POCD are complex and multifactorial. Risk factors include genetic and some somatic diseases as well as preoperative stress, but general anesthesia has been conclusively proven to be the major etiologic factor in the ISPOCD1 study[25]. In connection with these data, it is reasonable to question the use in everyday practice of effective and safe neuroprotective agents to face the negative effects of general anesthesia. These agents should act on the same systems that are suppressed by general anesthesia and interrupt the cascades of pathological reactions. According to the definition of The World Health Organization, nootropics are pharmacological agents that activate learning, improve memory and mental activity, while they increase the brain's resistance to aggressive hypoxia, trauma and intoxication. It may be justified to use the metabolite-tropic and endothelial protective drug Piracetam, which has a neuroprotective effect, as it has been proven in many studies[26,27]. Also, the drug Citicoline can be considered as a cerebroprotector, which inhibits the activation of phospholipases A1, A2, C and D, reduces the formation of free radicals, prevents the destruction of membrane systems, preserves antioxidant protective systems and inhibits apoptosis. Recently, studies have appeared on the cytokine mechanisms of the pathogenesis of anesthesia-induced neurodegradation[20,28]. Cytokine imbalance - the overproduction of IL-1β and the relative deficiency of its receptor antagonist IL-2 - has a great importance in brain damage. This allows us to consider IL-1 receptors as a promising target for neuroprotection in case of anesthesia-induced brain damage. Several studies have shown the neuroprotective activity of the interleukin-1 receptor antagonist (RAIL - IL-1 blocker) in experimental acute cerebrovascular accident, traumatic brain injury, and diabetic encephalopathy[19-21,28]. Recombinant RAIL-1 is a non-glycosylated analogue of an interleukin 1 which differs by one amino acid sequence in the N-terminal part from its native form. A new dosage form of RAIL - a gel for intranasal administration has been developed.

In this study, we first made attempts to interrupt the reaction of initiation of neuroapoptosis after ketamine, by the use of IL-1b antagonists - the active pharmacological agent RAIL in the form of an intranasal gel. The aim of the study was to evaluate the neuroprotective and anti-apoptotic activity of the IL-1 antagonist RAIL-gel in comparison with reference drugs Citicoline and Piracetam during ketamine anesthesia.

Materials and methods

Materials

Ketamine and Piracetam were purchased by PJSC FARMAK, Ukraine and Citicoline byTakeda, Japan. The substance RAIL was obtained from the Federal State Unitary Enterprise "State Scientific Research Institute of Highly Pure Biological Preparations" (Russia,

S-Petersburg, LSR-007452 / 1-0300710). Recombinant substances and RAIL in particular were obtained biotechnologically from microorganisms, protozoa and cereal. The test sample (RAIL) was obtained biotechnologically from E. coli TG1 (pTAC-hlL-1ra) and consists of 153 amino acids. Its molecular weight is 17, 906 kDa. RAIL-gel (5 mg / 1 ml) was developed at the Department of Technology of Drug of ZSMU. We also used paraplast (MkCormick, USA), bovine serum albumin solution (Sigma, USA, Cat. No. A2153), primary antibodies to the protein c-fos (Sigma Chemical, USA), secondary antibodies (fluorescent conjugated goat IgG - Sigma Chemical, USA), primary antibodies to bcl2 (Santa Cruz Biotechnology), solution of secondary antibodies (1: 1000) (biotinylated anti-mouse IgG, Sigma, USA, cat. No. 051M4885), ExtrAvidin-peroxidase solution (Sigma, USA, Cat. No. 051M4885), solution of AEC (1 tablet of 3-amino-9-ethylcarbazole (Sigma, USA, cat. No. a6926), solution of NaCl 0.9% (Ukraine), 30% H2O2 (Ukraine).

Animal experiments

The study used 50 white nonlinear rats at the age of 6 months from the nursery of the GA «Institute of Pharmacology and Toxicology of the National Academy of Medical Sciences of Ukraine». Weight of rats was 180-200 g. The acclimatization period was 14 days for all animals. The experimental studies were carried out in accordance with the "Regulations on the Use of Animals in Biomedical Research" and with the European Convention on the Protection of Animals Used for Scientific and Other Purposes. The experiment was approved by the Bioethics Committee of Zaporizhzhia State Medical University. Animals were kept under standard vivarium conditions with a change in the light cycle with an ambient temperature of 220 C. The manipulations were performed under ethanol-sodium anesthesia. Anesthesia was caused by intraperitoneal injection (IP injection) of ketamine at a dose of 100 mg/kg. Immediately after rats' emergence from anesthesia they were administered drugs at the following doses: Piracetam - 500 mg/kg, intraperitoneally, Citicoline - 500 mg/kg, intraperitoneally, RAIL-gel - 1 mg/kg intranasally using the dispenser pipette. The animals were divided into 5 groups (10 rats in each group): the first group - intact (control), the second - animals with experimental ketamine anesthesia. The third group - rats with ketamine anesthesia, which were administered Piracetam (PJSC FARMAK, Ukraine, 200 mg/ml) The fourth group received Citicoline (Takeda, Japan, 1000 mg / 4ml) after anesthesia. The fifth group received RAIL-gel after ketamine anesthesia. The intact group was injected intraperitoneally one time with a solution of NaCl 0.9% at the rate of 1 ml/0.1 kg, and the control group was administered the same dose of saline solution after ketamine anesthesia. The slaughter of rats was performed after 3 hours.

Instrumentation

Instruments used were the following: Buen clamp, rotary microtome Microm-325 (Microm Corp., Germany), refrigerated centrifuge Sigma 3-30k (Germany), fluorescence microscope Axioscop (Ziess, Germany), a high-sensitivity COHU4922 video camera (COCHU Inc., USA), HistoStar paraffin filling station (Thermo Fisher Scientific, USA), an automated PT-module (Thermo Scientific, USA), Red Line thermostats (Binder, Nimechchina), pH meter MP-220 (Mettler Toledo, Switzerland), installation for immunoblotting Mini Trans-Blot, Bio-Rad Laboratories (USA), thermostat TDB-120, torsion scales, set of batchers Bio HIT (Finland), Axiovision digital image analysis system (Carl Zeiss, Germany) with analysis programs and software development environment AZxioVision and Zen (Carl Zeiss, Germany).

Western blotting

The blood was quickly removed from the brain, separated from the meninges, and the test pieces were placed in liquid nitrogen. Then it was ground in liquid nitrogen to a powdery state and homogenized in a 10-fold volume of a medium at (2° C) containing (in mmol): sucrose - 250, Tris-HCl buffer - 20, EDTA -1 (pH 7.4). The mitochondrial fraction was isolated by differential centrifugation at a temperature +4° C. To purify the mitochondrial fraction from large cell

fragments, centrifugation was preliminarily carried out for 7 minutes at 1000g, and then the supernatant was re-centrifuged for 20 minutes at 17000g. The supernatant was decanted and stored at -80° C. The concentration in the cytoplasmic fraction of the brain bcl-2 was determined by Western blot analysis. Proteins were separated on a 10% polyacrylamide gel (PAGE). Separation of protein fractions was carried out by electrophoresis at a voltage of 100 V (for gel compaction), when the samples reached the interface 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 h. After transfer, the membrane was placed in a blocking buffer containing 1% bovine serum albumin solution for 20 hours. Washed on a shaker for 5 min in a solution of 0.1 M phosphate buffer (pH 7.4), the membrane was placed in a solution of primary antibodies against bcl-2 and incubated for 2 h at room temperature. Washed on a shaker 4 times for 5 minutes in 0.1 M phosphate buffer (pH 7.4). The membrane was placed in a solution of secondary antibodies (1: 1000), incubated for 2 hours. 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 in 1% bovine serum albumin solution (1: 1000). Incubated for 1 hour and washed. For visualization, the membrane was treated with AEK solution: 1 tablet of 3-amino-9-ethylcarbazole 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 incubated in the substrate mixture for 5-10 min. A red insoluble precipitate characterizes the antigen-antibody complex in the blot. The membrane was washed in distilled water several times. The strips were dried between sheets of filter paper under a flow of cold air. Detection of bcl-2 was carried out using densitometry in the Adobe Photoshop program.

Immunohistochemical studies

To detect the expression of c-fos in the CA1 zone of the hippocampus, an immunohistochemical method of indirect immunofluorescence was used. For histochemical studies, the brain was fixed in Carnoy's fluid for 24 hours and then embedded in paraplast according to the standard scheme. On a rotary microtome Microm-325 (Microm Corp., Germany), 15-micron sections of the CA1 hippocampus were made, which were dewaxed according to a standard technique. Histological sections of the brain were isolated from the paraplast and rehydrated, washed three times for 5 minutes with phosphate buffer (pH = 7.4) and incubated with 2n hydrochloric acid (T = 37° C) for 30 minutes. Then, each was washed twice for 5 minutes with phosphate buffer (pH = 7.4), twice for 5 minutes with borate buffer according to Holmes (pH = 8.4) and four times for 5 minutes with phosphate buffer (pH = 7.4), ~~after which incubated~~ followed by incubation for 30 minutes with 0.1% trypsin solution in phosphate buffer (T = 37° C). After incubation, the sections were washed four times for 5 minutes with phosphate buffer (pH = 7.4). First, primary antibodies to the c-fos protein were applied to the sections and incubated at +40° C for 24 hours. After incubation, the sections were washed three times with 0.1 M phosphate buffer. Then, secondary antibodies (fluorescent conjugated goat IgG) were applied to the samples and incubated at room temperature for 60 min. After incubation, the sections were washed with 0.1 M phosphate buffer (pH = 7.4). After the final four-fold washing with phosphate buffer, the sections were embedded in a mixture of glycerol-phosphate buffer (9:1). Fos-immunopositive neurons were examined using a fluorescence microscope. The image of fos-immunopositive neurons of the CA1-zone of the hippocampus, obtained on a microscope, using a highly sensitive video camera was introduced into the computer hardware and software system for digital image analysis VIDAS.

Statistical analysis

Statistical analysis was performed using the standard statistical package "STATISTICA® for Windows 6.0" (StatSoftInc., No. AXXR712D833214FAN5), "SPSS 16.0" and "Microsoft Office Excel 2003". The normality of distribution was evaluated by the Shapiro-Wilk criterion. When the results were consistent with the

Table 1: Density of c-fos-positive cells in the brain of rats with ketamine anesthesia under the influence of Piracetam, Citicoline and RAIL-gel (M ± m, n = 10)

Groups of animals	Density of c-fos-positive cells
Intact animals (n=10)	12.9 ± 2.0
Control – animals with ketamine anesthesia(n=10)	127.4 ± 5.0*
Animals with ketamine anesthesia +Piracetam(n=10)	116.4 ± 5.0*
Animals with ketamine anesthesia +Citicoline(n=10)	109.6 ± 7.6* [1]
Animals with ketamine anesthesia +RAIL-gel(n=10)	91.1 ± 4.6* [1,2]

**- Differences are significant at p < 0.05 compared to intact group; 1- Differences are significant at p < 0.05 compared to control group; 2- Differences are significant at p < 0.05 compared to group treated with Piracetam; n – number of animals in the group.*

Table 2: Concentration of bcl-2 protein in the brain of rats with ketamine anesthesia under the influence of Piracetam, Citicoline and RAIL-gel (M ± m, n = 10)

Groups of animals	Concentration of bcl-2 protein
Intact animals(n=10)	45.2 ± 2.3
Control – animals with ketamine anesthesia(n=10)	23.2 ± 2.3*
Animals with ketamine anesthesia + Piracetam(n=10)	24.4 ± 1.6*
Animals with ketamine anesthesia + Citicoline(n=10)	32.6 ± 4.8* [1,2]
Animals with ketamine anesthesia + RAIL-gel(n=10)	38.2 ± 3.9* [1,2]

**- Differences are significant at p < 0.05 compared to intact group; 1- Differences are significant at p < 0.05 compared to control group; 2- Differences are significant at p < 0.05 compared to group treated with Piracetam; n – number of animals in the group.*

law of normal distribution of the trait, the reliability was estimated by the Student's t-test. In the case of a distribution other than the normal one, the U Mann-Whitney criterion was used. For comparison of independent variables in more than two samples, ANOVA for normal distribution or Kruskal-Wallis criterion for non-normal distribution was used. P-values less than 0.05 (*p < 0.05) were considered statistically significant for all types of analysis.

Results and Discussion

As a result of the study, it was found that ketamine anesthesia is accompanied by the onset of signs of apoptosis of CA1 hippocampus neurons. These findings confirm the apoptotic effect of ketamine anesthesia by the increased expression of c-fos proteins (**Table 1**). The c-fos expression in the CA1 zone of the brain hippocampus increased by almost 10 times (p <0.01) in the control group of rats, treated with ketamine anesthesia compared with the intact group, indicating the activation of neuroapoptosis. In addition Ketamine significantly reduces the body's anti-apoptotic protection by inhibiting the bcl-2 protein in the brain (**Table 2**) and in combination with the above mentioned, increase in the expression of the c-fos rapid response protein in the CA1 area of the hippocampus, leads to programmed neuronal death. The use of neuroprotective drugs is considered to prevent the occurrence of cascades of such pathological reactions, reducing the expression of early response genes and activating anti-apoptotic protective mechanisms. Indeed, there was a decrease in the number of c-fos-positive neurons in rats treated with

piracetam as a neuroprotective agent after ketamine anesthesia by 8.6% (p <0.05), compared to the control group. There was a 14% (p <0.05) decrease in the amount of c-fos compared to controls in the group of animals with ketamine anesthesia treated with Citicoline, and a 25.5% (p <0.05) decrease in animals treated with RAIL-gel compared to the control group. Thus, the best stabilizing effect on the c-fos protein level in the CA1 zone of the rat brain hippocampus was demonstrated by RAIL-gel, which exceeded the anti-apoptotic effect of Citicoline by 11.5% and Piracetam by 16.9%. The effect of these neuroprotective agents on the level of anti-apoptotic protein bcl-2 is shown in Table 2. After ketamine anesthesia a decrease in bcl-2 level was observed in the control group by 48.7% (p <0.01), compared with the intact group. In rats treated with neuroprotective agents after ketamine anesthesia, the following results were observed:

The amount of bcl-2 protein decreased by 46% in the rat brain upon the use of Piracetam compared to the intact group, while it was 5% higher compared to the control group.

The decrease in amount of bcl-2 protein was 27.9% in the group of rats treated with Citicoline compared to the intact group, while its concentration was 28.8 higher compared to the control group.

- animals receiving RAIL-gel after ketamine showed a 15,4% decrease in the amount of bcl-2 in the homogenate compared to intact, and a 39.2% increase in its concentration compared to the control group.

The RAIL-gel showed the best results, both in reducing the density of c-fos-positive neurons and in the storage of anti-apoptotic protein bcl-2 under conditions of ketamine anesthesia compared to the nootropics piracetam and citicoline. These findings provide experimental justification for further in-depth study of RAIL as a potential neuroprotective agent in order to prevent the negative consequences of general ketamine anesthesia.

As shown in the study the anti-apoptotic and neuroprotective effect of RAIL-gel in ketamine-induced neurodegradation is associated with its ability to interrupt IL-1β-dependent mechanisms of neuroapoptosis. Namely, the RAIL-gel inhibits IL-1-dependent expression of iNOS in glial cells and reduces NO overproduction, inhibits the exit of mitochondria proapoptotic proteins. RAIL-gel can inhibit the expression of redox-sensitive apoptosis genes of early response, mainly JunD and c-fos, due to a decrease of NO levels [21,28]. Citicoline can have an anti-apoptotic and neuroprotective effect in case of anesthesia-induced brain damage due to the presence of a pronounced mitoprotective effect, which may underlie its anti-apoptotic activity. Apparently, Citicoline modulates the activity of cyclosporin A-dependent mitochondrial pore during ischemia and terminates the release of proapoptotic factors through it.

It has been shown that Citicoline can maintain the integrity of the inner mitochondrial membrane. A similar mechanism is associated with the restoration of cardiolipin levels in the inner mitochondrial membrane. In addition, it was found that citicoline indirectly, by increasing the activity of glutathione-linked enzymes (glutathione reductase and glutathione transferase), regulates the level of reduced glutathione and, thus, can reduce the level of cytoxic derivatives of NO[19,20,29-31]. Citicoline's ability to inhibit apoptosis by reducing the expression of procaspase is also known[31]. Piracetam did not show significant anti-apoptotic and neuroprotective effects in ketamine-induced neurodegradation. The mechanism of action of this nootropic is associated with the activation of anaerobic pathways of ATP synthesis in the brain, an increase in the level of acetylcholine and the concentration of n-cholinergic receptors. The membrane stabilizing and stress-protective properties of Piracetam are known. It is proven that Piracetam is ineffective in extreme conditions of the central nervous system, it can enhance lactic acidosis during cerebral ischemia. We found that Piracetam enhances anxiety and does not affect cognitive-mnestic disorders after ketamine anesthesia[9,20,26-29,38].

Conclusions:

The neurodegradative effect of ketamine anesthesia (100 mg / kg) was demonstrated in the reproduced experiment by activating apoptosis processes (in-

creased density of c-fos positive cells in the CA1 area of the hippocampus) and inhibiting the organism protective capacity (decreased amount of bcl-2 protein in the brain).

The use of the neuroprotective agents such as Piracetam, Citicoline, and RAIL-gel in rats treated with general anesthesia was found to lead to a decrease of the neurodegradative effect of ketamine, as reflected in a decrease in the concentration of c-fos protein and an increase in the expression of bcl-2 protein.

The best neuroprotective effect was demonstrated by the RAIL-gel. It had the most significant effect on the expression of c-fos and bcl-2 proteins, reducing the first one and activating the second one, compared to other drugs. The second most effective drug is Citicoline, followed by Piracetam. □

Ethical considerations

All investigations conformed to the ethical of research and were approved by the Bioethics Committee of Zaporizhzhia State Medical University (prot. No. 2 of 04/14/2015) and the authors of this manuscript observed ethical issues. Animals were handled according to the International Guidelines for Care and Handling of Experimental Animals.

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Conflict of interest:
The Authors declare no conflict of interest.

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