INTENSITY OF ENDOPLASMIC RETICULUM STRESS, AUTOPHAGY, AND APOPTOSIS IN THE CEREBRAL CORTEX OF RATS WITH CHRONIC ETHANOL CONSUMPTION UNDER THE INFLUENCE OF THE COMPLEX COMPOUND OF GERMANIUM WITH NICOTINIC ACID

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The aim of the research – to determine the level of BAX, caspase-3, GRP78, IRE1 and Beclin-1 in the cerebral cortex of rats with chronic ethanol consumption and under conditions of exposure to the germanium complex with nicotinic acid (MIGU-1).

Materials and methods. Female rats had free access to 20 % C2H5OH as the only source of fluid for 110 days. Starting from the 90th day, the animals were injected with MIGU-1 (10 mg/kg/day, IP). The expression level of BAX, caspase-3, GRP78, IRE1 and Beclin-1 in the tissue was determined by Western blot analysis.

Results. In rats with chronic ethanol consumption, the level of BAX-dimer increased by 2.06 times (p<0.001). The introduction of MIGU-1 caused a decrease in the level of BAX-dimer by 1.42 times (p<0.05). In rats with chronic ethanol consumption, the level of caspase-3 increased by 2.12 times (p<0.05), cleaved caspase-3 increased by 6.37 times (p<0.05). When MIGU-1 was administered, the level of caspase-3 decreased by 1.73 times (p<0.05). Under the conditions of MIGU-1 administration, protein bands of cleaved caspase-3 were reduced to an undetectable level. In rats with chronic ethanol consumption, the level of GRP78 increased by 1.72 times (p<0.05). After administration of MIGU-1, no changes in the level of GRP78 were recorded. Long-term ethanol consumption increased the levels of IRE1 by 1.74 times (p<0.05) and p-IRE1 by 2.7 times (p<0.001). In the presence of MIGU-1, the levels of IRE1 and p-IRE1 did not change. Under the conditions of chronic ethanol consumption, an increase in the levels of Beclin-1 by 2.33 times (p<0.001) and p-Beclin-1 by 4.69 times (p<0.001) was observed. Administration of MIGU-1 did not affect the level of Beclin-1, while the level of p-Beclin-1 decreased by 3.09 times (p<0.001).

Conclusions. Long-term ethanol consumption triggers metabolic changes in the cerebral cortex, resulting in ER stress, UPR activation, autophagy, and apoptosis. Administration of MIGU-1 alleviates ER stress by selectively inhibiting specific branches of apoptosis through effects on Beclin-1 levels, suggesting an effect of MIGU-1 on neuronal survival under chronic ethanol consumption

Keywords: apoptosis, autophagy, chronic alcohol consumption, coordination compound of germanium


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3.cio-economic factors can become a systemic disease. In view of this, understanding the role and regulation of the above-mentioned intracellular processes in maintaining cellular homeostasis in the nervous tissue of rats can provide a new understanding of the pathogenesis of neuropsychiatric, inflammatory, metabolic and dystrophic disorders under conditions of chronic ethanol consumption, the emergence of new therapeutic targets in the treatment of chronic alcoholism.

The aim of the experiment was to investigate the role of ER stress marker proteins, the unfolded protein response (UPR), autophagy and apoptosis in the cerebral cortex of rats with chronic ethanol consumption and the possible influence of the coordination compound germanium with nicotinic acid (MIGU-1) on these processes.

2. Research planning (methodology)

Based on the results of previous studies, the coordination compound of germanium with nicotinic acid (MIGU-1), which overcomes the blood-brain barrier [9] and can reduce the level of oxidative stress under conditions of toxic effects of various etiologies, such as the syndrome of prolonged crushing [10], toxic effects of anthracycline antibiotics [11, 12], etc.

Stages of the research:
1) analysis of modern research data;
2) simulation of chronic ethanol intoxication;
3) introduction of a coordination compound of germanium with nicotinic acid;
4) Western blot analysis;
5) processing and analysis of the obtained results;
6) determination of prospects for further research.

3. Materials and methods

The study was carried out within the framework of the research topic of the Bogomolets National Medical University “Search and study of pharmacological properties of germanium coordination compounds and derivatives of heterocycles”, implementation dates 2021-2023, state registration number 0121U109363.

The study was performed on female Wistar rats weighing 197.7±3.1 g in the autumn-winter period. All manipulations with animals were carried out in compliance with the requirements of the “European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes” [13]. The animals were kept in the vivarium of the Bogomolets National Medical University (Kyiv, Ukraine) with compliance with biochemical norms when conducting experimental research on animals, which was confirmed by the Commission on Bioethical Expertise and Ethics of Scientific Research of Bogomolets National Medical University (protocol No. 177 dated 23.10.2023).

For the experiment, rats were selected according to the results of the two-bottle alcohol choice test (20 % ethanol) [14]. Rats were divided into 3 groups: group I – intact animals (n=6), which had free access to a balanced diet and water, group II – animals with chronic ethanol use, which had free access to a balanced diet and 20 % ethanol as the only liquid source (n=6), group III – experimental group (n=6). The experimental group consisted of animals from group II, which were injected intraperitoneally with a coordination compound of germanium with nicotinic acid MIGU-1 at a dose of 10 mg/kg/day from the 90th day until the end of the experiment [15]. The experiment was conducted for 110 days. Animals were decapitated under light ether anesthesia, the cerebral cortex was immediately removed and frozen in liquid nitrogen.

The content of apoptosis, autophagy, and endoplasmic reticulum stress proteins in the cerebral cortex of rats was determined using Western blot analysis [16]. Brain proteins were extracted with RIPA Lysis Buffer with the addition of protease inhibitor cocktail (PIC) (Sigma, USA) for 20 minutes in an ice bath. The homogenate was centrifuged at 16,000 g for 20 minutes at a temperature of 4 °C. The concentration of total protein in the supernatant was measured using the “Total protein” test system (High Technology, USA) on a semi-automatic biochemical analyzer BioChem SA (High Technology, USA). Lysate proteins (50 μg/track) were separated into fractions by the SDS PAGE method in a BioRad (USA) vertical chamber, using a Tris-glycine buffer (pH 8.3) at 50 W (concentration of samples), 160 W – sample separation, according to the U.K. method. Laemmli [17]. Proteins from PAGE were transferred to a nitrocellulose membrane with a pore diameter of 0.45 μm (GE Healthcare, “Amersham”, Great Britain) for 60 minutes at a temperature of 4 °C. After transfer, the membranes were blocked with a 5 % solution of nonfat dry milk (Carnation, USA) in PBST for 2 hours at room temperature. After blocking, the membranes were incubated with specific antibodies against BAX (Bel2 Associated X Protein) (1:500, Invitrogen, USA, #UA2671705), caspase-3 (1/2500, Abcam, USA # ab208161), Glucose-Regulated Protein 78 (GRP78 ) (1:1000, #PA5-34941, Invitrogen, USA); Inositol-requiring enzyme type 1 (IRE1) (1:800, #PA5-79193, Invitrogen, USA); Beclin-1 (1:1500, Invitrogen, USA); Protein 78 (GRP78 ) (1:1000, #PA5-34941, Invitrogen, USA); Inositol-requiring enzyme type 1 (IRE1) (1:800, #PA5-79193, Invitrogen, USA); Beclin-1 (1:1500, Invitrogen, USA); Protein 78 (GRP78 ) (1:1000, #PA5-34941, Invitrogen, USA); Inositol-requiring enzyme type 1 (IRE1) (1:800, #PA5-79193, Invitrogen, USA); Beclin-1 (1:1500, Invitrogen, USA); and beta Actin (1:5000, Loading Control Monoclonal Antibody (BA3R) (#MA5-15739, Invitrogen, USA) in PBST for 16 hours at 4 °C. After incubation, the membranes were washed in RBST 6 times for 5 minutes each and incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase for 90 minutes at room temperature. Non-specifically adsorbed secondary antibodies were washed with RBST 6 times. The specific antigen-antibody complex was detected by the enhanced chemiluminescence (ECL) method. Visualization of specific staining was carried out on Konica Minolta X-ray films (Medical & Graphic, Inc, Japan). The scanned immunoblotting results were subjected to densitometry using the TotalLab TL120 program (Nonlinear Inc., USA). The relative content of proteins was expressed in conventional units of optical density (arbitrary units – a.u.).

Statistical processing of the results was performed using the licensed statistical package IBM SPSS Statistics, version 23.0 (SPSS Inc., USA), Prism 9.5.1 program (GraphPad Software Inc., USA). Data were tested for normality using the Shapiro-Wilk test. For normally dis-
tributed data, statistical differences between groups were analyzed by one-way ANOVA test. Arithmetic mean (M) and mean error (+SEM) were determined for indicators whose distribution was normal. Mathematical processing of the results was carried out using an unpaired t-test. The difference between the data in the compared groups was considered statistically significant at a probability equal to 95% ($p<0.05$).

4. Results

Apoptosis, autophagy, and the unfolded protein response to ER stress are important cellular processes that ensure the proper functioning of the body under stress conditions. Changes in the levels of the pro-apoptotic protein BAX and the effector enzyme caspase-3, which plays an important role in apoptosis, were investigated using Western blot analysis; chaperone ER stress indicator GRP78, also called HSPA5 (Heat Shock Protein Family A (Hsp70) Member 5), protein IRE1 – serine/threonine kinase, which acts as one of the three branches of the UPR signalling pathway, and the autophagy protein Beclin-1 in the cerebral cortex of rats with chronic ethanol consumption and intraperitoneal administration of the coordination compound germanium with nicotinic acid MIGU-1 against the background of chronic alcohol consumption.

A sign of the activation of apoptosis is the oligomerization of the BAX molecule from a monomer to a dimer, which structures pores in the outer membrane of mitochondria for the release of cytochrome c and other apoptogenic factors from the intermembrane space of mitochondria into the cytosol [18]. In our work, the level of BAX dimer in the cerebral cortex of animals with chronic consumption of ethanol was 2.06 times higher than in intact rats ($p<0.001$) (Fig. 1). This result confirms the state of oligomerization of BAX and the initiation of a cascade of further events, which ultimately lead to the activation of caspase-3.

Administration of MIGU-1 contributed to a 1.42-fold decrease in the level of BAX-dimer in the cerebral cortex of experimental group animals compared to animals with chronic ethanol consumption ($p<0.05$). However, the level of BAX-dimer in the examined tissue of these rats was 1.45 times higher than that of intact animals ($p<0.05$).

An important marker of the apoptotic activity of cells is the assessment of the content of caspase-3. The results of Western blot analysis of the level of caspase-3 (37 kDa) and cleaved caspase-3 (17 kDa) in the cerebral cortex of animals are presented in Fig. 2.

The study of caspase-3 in the cerebral cortex of rats under conditions of chronic use of a 20% ethanol solution showed an increase in its level by 2.12 times in relation to the group of intact animals ($P=0.0450$). It is known that apoptosis occurs precisely because of the activation of caspase enzymes. Therefore, as can be seen in Fig. 2, immunoblotting revealed cleavage of caspase-3 in the cerebral cortex of rats with chronic ethanol consumption. The level of cleaved caspase-3 in the cerebral cortex of these animals was 6.37 times higher than in intact animals ($p<0.05$). The increased content of cleaved caspase-3 is evidence of the activation of programmed cell death (apoptosis) under conditions of long-term use of 20% ethanol.

Administration of MIGU-1 against the background of 20% ethanol consumption reduced the level of caspase-3 in the cerebral cortex by 1.73 times compared to the group of animals with chronic ethanol consumption ($p<0.05$). There were no significant changes in the cerebral cortex of experimental animals in relation to the level of caspase-3 in the examined tissue of intact animals ($p>0.05$). Analysis of the immunoblotogram in Fig. 2, a shows that the protein band of cleaved caspase-3 in the group of experimental animals is reduced to an undetermined level. This proves the inhibitory effect of MIGU-1 on the activation of caspase-3 in the cerebral cortex.

Fig. 1. The level of BAX-dimer in the cerebral cortex of rats under normal conditions (intact animals), chronic ethanol consumption (20% ethanol) and MIGU-1 administration (20% ethanol+MIGU-1): a – representative immunoblotogram; b – quantitative determined the level of BAX-dimer in lysates of the cerebral cortex relative to the level of beta-actin (as a control); * – $p<0.05$ compared to intact animals, # – $p<0.001$ compared to intact animals; ** – $p<0.05$ compared to animals with chronic use of 20% ethanol.
To find out the further directions of caspase-3 activation in the cerebral cortex of rats with chronic ethanol consumption, the level of expression of the main endoplasmic reticulum stress marker GRP78, also known as BiP (Binding Immunoglobulin Protein) [19]. In addition, GRP78 acts as a regulator of apoptosis was evaluated [20]. Our results showed a 1.72-fold increase in the level of GRP78 in the cerebral cortex of rats with chronic ethanol consumption compared to intact animals \( (p<0.05) \) (Fig. 3).

An increase in the level of GRP78 in the cerebral cortex confirmed the presence of ER stress in the nervous tissue of rats under the influence of 20 % ethanol under conditions of long-term consumption.

It was shown that the introduction of MIGU-1 did not affect the level of GRP78 in the cerebral cortex of animals \( (p=0.3846) \). The level of GRP78 in the experimental group was 1.55 times higher than that of the group of intact animals \( (p<0.05) \).

During ER stress, GRP78 dissociates from transmembrane stress sensor proteins: ATF6 (activating transcription factor 6), PERK (PKR-like endoplasmic reticulum (ER) kinase) and IRE1, activating each of them and the unfolded protein response (UPR) [21]. The IRE1 protein plays a key role in the UPR, the purpose of which is to restore the normal state of the endoplasmic reticulum and ensure cell survival under stress conditions. Phosphorylation of IRE1 is an important regulatory mechanism that controls the activity of this protein and its participation in the cell’s response to ER stress [22]. The Western blot analysis method allowed us to determine the levels of dephosphorylated IRE1 protein (110 kDa) and the phosphorylated form of p-IxRE1 (150 kDa) in the cerebral cortex of rats. The results of IRE1 and p-IRE1 immunoblotting are shown in Fig. 4.

Long-term use of ethanol with a concentration of 20 % increased the levels of IRE1 in the cerebral cortex of animals by 1.74 times \( (p<0.05) \) and p-IRE1 by 2.7 times \( (p<0.001) \).

Administration of MIGU-1 did not affect the level of dephosphorylated and phosphorylated IRE1 protein in the cerebral cortex of experimental rats compared to animals with chronic ethanol consumption \( (p>0.05) \) and p-IRE1 by 2.7 times \( (p>0.05) \).

Analysis of the IRE1/p-IRE1 ratio (Fig. 4, d) in the cerebral cortex of animals showed that under the conditions of long-term use of 20 % ethanol and the combina- tion of 20 % ethanol with MIGU-1, this indicator was lower compared to intact animals \( (p<0.05\) and \( p<0.05 \) respectively). At the same time, administration of MIGU-1 probably did not affect the ratio of IRE1/p-IRE1 in studied animals with chronic ethanol consumption \( (p>0.05) \).
Currently, the mechanism of IRE1 activation is not fully understood, but it is believed that IRE1 can be activated by triggering various signalling pathways, including apoptosis and autophagy [23].

In addition, in our work, the level of the Beclin-1 protein, which is involved in the regulation of autophagy, a process that is important for maintaining cellular homeostasis and various physiological and pathological processes, including neurodegeneration, was investigated using Western blot analysis [24]. Phosphorylated form of Beclin-1 also participates in such cellular processes as apoptosis and regulation of cellular metabolism [25].

The results of determining the content of dephosphorylated Beclin-1 (60 kDa) and phosphorylated p-Beclin-1 (75 kDa) in the cerebral cortex of rats using Western blot analysis are shown in Fig. 5.
Consumption of 20 % ethanol for 110 days caused a 2.33-fold increase in the level of Beclin-1 and a 4.69-fold increase in the level of p-Beclin-1 in the cerebral cortex of rats compared to intact animals (\( p < 0.001 \) and \( p < 0.001 \), respectively). Such changes in the Beclin-1 protein level in the studied tissue of rats indicate the activity of autophagy processes as an important protective mechanism for compensating for metabolic shifts that occurred under the conditions of long-term ethanol consumption.

Against the background of 20 % ethanol consumption, intraperitoneal administration of MIGU-1 probably did not affect the level of Beclin-1 in the cerebral cortex of rats compared to intact animals (\( p > 0.05 \) and \( p > 0.05 \), respectively). Such changes in the Beclin-1 protein level in the studied tissue of rats indicate the activity of autophagy processes as an important protective mechanism for compensating for metabolic shifts that occurred under the conditions of long-term ethanol consumption.

As can be seen in Fig. 5, \( d \), long-term administration of 20 % ethyl alcohol and administration of MIGU-1 against this background did not affect the ratio of Beclin-1/p-Beclin-1 in the cerebral cortex compared with intact animals (\( p > 0.05 \)). However, administration of MIGU-1 increased the ratio of Beclin-1/p-Beclin-1 by 2.69 times compared to the group of animals with chronic ethanol consumption (\( p < 0.001 \)) to the level of the group of intact animals.

5. Discussion of research results

Current views on the pathogenesis of neuronal damage due to chronic ethanol use indicate the involvement of a large number of cellular mechanisms, including inflammation, induction of oxidative stress, damage to DNA, proteins and lipids.

Our previous study showed that chronic consumption of 20 % ethanol for 110 days caused the activation of neuroglial cells in the brain of rats. Under these conditions, the effect of MIGU-1 when administered intraperitoneally to rats with chronic ethanol consumption was manifested in inhibited activation of neuroglia [26]. According to the literature, during activation, anti-inflammatory cytokines, and neurotrophic factors, which contribute to the recovery of neurons after damage, begin to be produced and released. However, in pathological conditions, the activation of neuroglia can have negative consequences, such as a gradual decrease in the number...
of neurons and functions related to memory, movement, and other neural processes [27]. This information allowed us to evaluate the presence of a neuroprotective effect of MIGU-1 on the cellular processes occurring in the cerebral cortex of rats with chronic ethanol consumption, and the ways through which the neuroprotective effect of MIGU-1 can be realized. In this study, we aimed to find out which intracellular signalling pathways may be involved in the neuroprotective effect of MIGU-1.

The results of the experiment revealed that during the long-term use of 20% ethanol in the cerebral cortex of rats, the processes of apoptosis, ER stress and autophagy become active, which reflects an increase in the levels of the pro-apoptotic protein BAX and the effector enzyme of apoptosis caspase-3, the chaperone GRP78 – the main endoplasmic reticulum stress marker, which controls the activation of UPR signaling, IRE1 – one of the proteins of the UPR system and the main autophagy regulator Beclin-1. The Western blot method showed significant levels of cleaved caspase-3, p-IRE1 and p-Beclin-1 (6.37 times, 2.7 times and 4.69 times) in the cerebral cortex of animals with chronic ethanol consumption compared to the group intact animals.

Administration of MIGU-1 to animals on the background of 20% ethanol consumption markedly alleviated the programmed death of nerve cells due to a decrease in the expression level of caspase-3 to the values of the group of intact animals and the BAX-dimer by 1.42 times compared to animals with chronic ethanol consumption. To our knowledge, this is the first report that MIGU-1 alleviates apoptosis induced by chronic ethanol consumption by regulating mitochondrial dysfunction and inhibiting the caspase cascade. In our opinion, the possible antiapoptotic effect of MIGU-1 is due to the ability of nicotinic acid as a component of the complex to reduce the level of oxidation of lipids [28] and proteins [29] and the properties of the entire complex to restore energy metabolism [30] and other possible mechanisms of action of germanium compounds [31].

UPR activation is an important mechanism required by cells to maintain protein and endoplasmic reticulum homeostasis, especially in neural tissues [32].

In our study, when MIGU-1 was administered intraperitonally, there were no significant changes in the levels of the components of the UPR system (GRP78, IRE1, p-IRE1) in the neurons of the brain of animals of the experimental group compared to animals with chronic ethanol consumption. However, in the cerebral cortex of experimental animals, a high level of GRP78 remained (1.55 times higher than the value in the group of intact animals), which may indicate the presence of ER stress. However, under the conditions of intraperitoneal administration of MIGU-1, the level of the transmembrane UPR-sensor non-phosphorylated protein RE1 in the cerebral cortex of experimental animals was probably not different, and p-IRE1 was 2.4 times higher compared to intact animals. Taking into account the facts that GRP78 expression blocks caspase activation [33, 34], and the IRE1 protein can interact with the pro-apoptotic BAX protein [35], it is logical to assume that the high levels of the GRP78 chaperone and p-IRE1 maintained in the presence of MIGU-1, help restore endoplasmic reticulum function and relieve stress by selectively inhibiting specific branches of apoptosis.

Although autophagy and apoptosis are distinct cellular processes, often with opposite outcomes, their signalling pathways are largely interconnected through various cross-talk mechanisms [36]. The physiological significance of the crosstalk between autophagy and apoptosis is poorly understood, but it is thought to contribute to a controlled and well-balanced cellular response to a particular stress signal. It is believed that during stress, the cell can choose between autophagy and apoptosis, depending on the intensity and duration of the stress exposure [37]. In our study, the high level of Beclin-1 in the cerebral cortex demonstrates the high activity of autophagy, as an important protective mechanism to compensate for the metabolic changes occurring in the nervous tissue during chronic ethanol consumption. Administration of MIGU-1 significantly reduced the level of p-Beclin-1 in the cerebral cortex of animals in the experimental group to the level of the group of intact animals. However, a high level of Beclin-1 was maintained in the cerebral cortex of animals in the experimental group compared to intact animals. Such changes in the Beclin-1 protein level in the presence of MIGU-1 were reflected in the shift of the Beclin-1/p-Beclin-1 ratio toward dephosphorylated Beclin-1. Therefore, the ability of MIGU-1 to facilitate apoptosis of rat brain cells with chronic ethanol consumption is related to its effect on the level of Beclin-1, since Beclin-1 is known to act as a platform for complex formation during autophagy and also to be a bridge for the interaction between autophagy and apoptosis [38].

Practical significance. The obtained results add the understanding of the biochemical mechanism of the formation of ethanol addiction and its possible pharmacological correction with germanium coordination compounds.

Study limitations. In this work, the mechanisms of influence of MIGU-1 in rats with acute ethanol intoxication were not investigated.

Prospects for further research. Study of the intensity of endoplasmic reticulum stress, autophagy and apoptosis in other organs and systems in rats with chronic ethanol consumption.

6. Conclusions

In this study, we found that 20% ethanol consumption for 110 days triggers metabolic changes in the cerebral cortex that result in endoplasmic reticulum stress, activation of the unfolded protein UPR response, and activation of autophagy and apoptosis pathways. Administration of MIGU-1 reduces the severity of the neurotoxic effects of chronic 20% ethanol consumption in the brain of rats. The mechanism of MIGU-1’s neuroprotective action may be to reduce the level of apoptosis-inducing BAX and caspase by inhibiting Beclin-1 phosphorylation and mediating the IRE1 signalling pathway in response to endoplasmic reticulum stress in the cerebral cortex of rats. Further study of the intracellular mechanisms by which MIGU-1 can affect the viability of brain cells under conditions of chronic etha-
nol consumption may be a promising step towards the development of new therapeutic strategies for the treatment of neurotoxic effects, neuropsychiatric disorders, and neurodegenerative diseases.

Conflict of interest
The authors declare that they have no conflict of interest in relation to this study, including financial, personal, authorship, or any other, that could affect the study and its results presented in this article.

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Data availability
Data will be provided upon reasonable request.

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