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ANALYSIS OF FACTORS INFLUENCING APOPTOTIC PROCESSES DURING FORMATION OF LONG-TERM HEALTH EFFECTS OF SEVERE ACUTE POISONING WITH NEUROTROPIC TOXICANTS

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Background. The nervous system damage caused by neurotoxicants is characterized by various morphological changes, manifested mainly by dystrophic and necrotic processes. The key mechanisms of post-intoxication asthenia pathogenesis, determined by the specifics of the toxicant, involve activation of apoptosis, trophic disorder, lipid peroxidation (LPO), neuropeptide regulatory insufficiency, as well as cerebrospinal fluid dynamics disorders.

Objective. Quantification of the contribution of apoptosis, oxidative stress, and neurotrophin regulation processes to the formation of long-term health consequences of severe acute poisoning with neurotropic toxicants.

Material and methods. Experimental studies were performed in male rats. The following toxicants were used: phenylcarbamate (1.6 mg/kg bw), methanol (11.5 g/kg bw), lead acetate (300 mg/kg bw). The period of formation of long-term health effects was 30 days. The level of apoptosis of the brain temporal cortex neurons was evaluated by the TUNEL method. The identification of blood plasma neurospecific proteins was carried out by the ELISA method. Evaluation of LPO and antioxidant system was carried out by standard biochemical methods.

Results. Exposure to the substances caused the signs of toxic effects in rats on days 1–2. The maximum severity of poisoning with phenylcarbamate was on the first day, while the maximum severity of poisoning with methanol and lead acetate was manifested on the second day. By days 5–7, the survived animals showed a normalization in the status regardless of the toxicant. On day 30, violations were detected, the totality of which allowed the survived animals to be divided into subgroups according to the manifestation of functional signs of long-term health effects of acute poisoning.

Conclusions. The formation of long-term health effects of severe acute poisoning with the studied neurotoxicants was shown to be associated with an increase in the number of TUNEL positive neurons and a decrease in the S100 protein serum concentration. Lipid peroxidation in brain tissues during the specified period did not play a significant role in apoptosis activation.

Keywords: psychoorganic syndrome; antioxidant system; apoptosis; neuron-specific proteins; neurotoxicants, late effect of intoxication; lipid peroxidation

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АНАЛИЗ ФАКТОРОВ, ВЛИЯЮЩИХ НА ПРОЦЕССЫ АПОПТОЗА В ПЕРИОДЕ ОТДАЛЕННЫХ ПОСЛЕДСТВИЙ ТЯЖЕЛЫХ ОСТРЫХ ОТРАВЛЕНИЙ НЕЙРОТРОПНЫМИ ТОКСИКАНТАМИ

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Введение. Поражение нервной системы при действии нейротоксикантов характеризуется различными морфологическими изменениями, проявляющимися в основном дистрофическими и некротическими процессами. Ключевыми механизмами патогенеза постинтоксикационной астении, определяющимися спецификой токсиканта, являются активация апоптоза, нарушение трофики, активация перекисного окисления липидов (ПОЛ), недостаточность нейропептидного звена регуляции, нарушения ликвородинамики.

Цель. Количественная оценка вклада процессов апоптоза, оксидативного стресса и нейротрофиновой регуляции в формировании отдаленных последствий тяжелых острых отравлений нейротропными токсикантами.

Материалы и методы. Экспериментальное исследование выполнено на крысах-самцах. В качестве токсикантов использовались: фенилкарбамат (1,6 мг/кг м.т.), метанол (11,5 г/кг м.т.), ацетат свинца (300 мг/кг м.т.). Период формирования отдаленных последствий составил 30 суток. Уровень апоптоза нейронов височной коры головного мозга определяли методом TUNEL. Определение нейроспецифических белков в плазме крови осуществлялось ИФА-методом. Определение показателей ПОЛ и антиоксидантной системы проводили стандартными биохимическими методами.

Результаты. Воздействие веществ вызывало признаки токсического действия у крыс на 1–2-е сутки, причем у фенилкарбамата с максимальной выраженностью в первые, а у метанола и ацетата свинца — на вторые. К 5–7-м суткам у выживших животных (независимо от токсиканта) состояние нормализовалось. На 30-е сутки были выявлены нарушения, совокупность которых позволила разделить выживших животных на подгруппы по уровню сформированности функциональных признаков отдаленных последствий острых отравлений.

Выводы. Показано, что формирование отдаленных последствий тяжелых острых отравлений для исследуемых нейротоксикантов связано с повышением количества TUNEL-позитивных нейронов и снижением концентрации белка S100 в сыворотке. Процессы ПОЛ в тканях мозга в указанные сроки не играли существенной роли в активации апоптоза.

Ключевые слова: психоорганический синдром; антиоксидантная система; апоптоз; нейроспецифические белки; нейротоксиканты; отдаленные последствия отравлений; перекисное окисление липидов

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INTRODUCTION

The long-term period following severe acute poisoning with neurotropic toxicants is characterized by the development of mainly nonspecific manifestations of the psychoorganic syndrome, post-intoxication and cerebrogenic asthenia, toxic polyneuropathies. Toxic damage to the nervous system, as well as to other body tissues, involves various morphological changes at the cellular and tissue levels and is manifested by various dystrophic and necrotic processes [1–2]. The use of light microscopy makes it possible to establish the presence of neuronal apoptosis, which is a consequence of most toxic lesions of the nervous system [3]. Toxic damage to brain tissue is characterized by small foci of necrosis, often elective, in which only individual tissue elements are damaged (some of them remain); such foci are manifested by rarefaction of the neuropil and gliopenia. The morphological basis of the psychoorganic syndrome in neurointoxication consists in the death of neurons and glial cells associated with both the direct toxic effect of xenobiotics and the induction of apoptotic processes [4]. The key mechanisms behind the pathogenesis of post-intoxication asthenia, determined by the specifics of the toxicant, involve activation of apoptosis, trophic disorder, lipid peroxidation (LPO), neuropeptide regulatory insufficiency, and cerebrospinal fluid dynamics disorders. Toxic neuropathies are manifested by segmental demyelination (toxic myelopathy) and axonal degeneration (toxic distal axonopathy).

The nonspecific mechanisms of toxic action characteristic of all neurotropic xenobiotics include inhibition of enzyme activity due to the blockade of sulfhydryl, carboxyl, amino, and other functionally active structural groups in peptides and proteins; formation of oxidative stress with subsequent activation of LPO processes[1]; mitochondrial inhibition; calcium homeostasis disorder; excitotoxicity; proinflammatory cytokine expression, inflammatory process induction in nervous tissue; haptenic modification of proteins with their acquisition of antigenic properties and induction of autoimmune damage mechanisms; inhibition of neurotrophin release, neurogenesis and gliogenesis in combination with suppression of proliferation and differentiation of new neurons and gliocytes; increased permeability of the blood-brain barrier; increased processes of cell apoptosis of the central and peripheral nervous system[2] [5–8].

Taking all the above mentioned into account, a deeper understanding of the long-term health effects of severe acute poisoning with neurotropic toxicants is required. In this connection, the present study aims to assess the quantitative contribution of apoptosis, oxidative stress, and neurotrophin regulation processes to the formation of long-term health consequences of severe acute poisoning with neurotropic toxicants.

MATERIALS AND METHODS

The experimental study involved 53 male outbred rats with a body weight (bw) of 180–220 g (baseline), received from the Kurchatov Institute Research Center — Rappolovo Nursery (Leningrad Region, Russia).

Long-term effects were simulated by a single administration of a neurotropic toxicant to laboratory animals at a dose of LD_{50}. The period of formation of long-term consequences was 30 days. The following were used as toxicants:

- phenylcarbamate, a reversible acetylcholinesterase inhibitor, synthesized in the Golikov Research Center of Toxicology [11];
- methanol (Vecton, Russia), an organic solvent that implements its toxic effect by damaging cell membranes;
- lead acetate (Reahim, Russia), an organic heavy metal salt that suppresses the activity of various enzymes.

Laboratory animals were divided into four groups:

- group 1 or C (Control) (n = 8), intragastric administration of 0.5 mL of saline solution;
- group 2 or PC (Phenylcambamate) (n = 15), intraperitoneal administration of an 0.1% aqueous solution at a dose of 1.6 mg/kg bw;

[1] FMBA Guidelines 12.08-2021 *Clinic, diagnosis and treatment of chronic poisoning (exposure) to neurotoxic substances*. 2021. (In Russ.)
[2] Badalyan AV., Belova MV., Brusin KM. et al. *Medical Toxicology: National guidelines.* M.: GEOTAR-Media, 2014. (In Russ.)

- group 3 or M (Methanol) (n = 15), intragastric administration of a 75% aqueous solution at a dose of 11.5 g/kg bw;
- group 4 or LA (Lead acetate) (n = 15), intraperitoneal administration of a 5% aqueous solution at a dose of 300 mg/kg bw.

The signs of toxic effects identified as a result of observation were evaluated in points according to the scale of signs of intoxication [10]. The dynamics of the animals' body weight, their consumption of feed and water were determined weekly.

To assess the formation of the phase of long-term consequences, neurophysiological testing of animals was performed on days 7, 15, 28 by such tests as open field (VideoMot 2, TSE, Germany), rotating rod (Rota-Rod Treadmills for rats 7700-7750, Ugo Basile, Italy), grip strength (Gripstrengthmeter 303500 series, TSE, Germany), sensory reactions tests (Startle Response System, TSE, Germany), assessment of the conditioned passive avoidance reflex (CPAR) (PACS-30, Columbus Instruments, USA).

On day 28, blood and brain tissue samples were collected from the survived animals (n = 32) for subsequent examination.

The determination of neurospecific proteins (neurospecific enolase NSE, brain neurotrophic factor BDNF, basic myelin protein MBP and calcium-binding protein S100) in animal blood plasma was carried out by enzyme immunoassay using commercial kits (Cloud-Clone Corp., USA) according to the manufacturer's instructions. Indicators of lipid peroxidation (diene conjugates of DC, malonic dialdehyde MDA); the antioxidant system of AOS (reduced glutathione GSH, activity of glutathione transferase GST, glutathione peroxidase GP, superoxide dismutase SOD), as well as energy metabolism (activity of glucose-6-phosphate dehydrogenase G6PDH) were determined in the homogenate of brain tissue, as described in [11].

The activity of apoptotic processes was assessed by determining the number of TUNEL-positive (terminal deoxynucleotidyl transferase dUTP nick end labeling) cells in the temporal cortex of rats. The choice of this area of the cortex was determined by the peculiarities of its cytoarchitectonics (high density of neurons, radial divergence of cortical columns, presence of eight types of interneurons and projections from the auditory, statokinetic, gustatory and olfactory analyzers, thalamus, their high sensitivity to hypoxia, frequent dysplasia and the ability to epileptogenesis). Apoptotic TUNEL cells were counted on 4–5 slices of the studied brain area using a commercial Elabscience® E-CK-A320 kit "TUNEL In Situ Apoptosis Kit (Green, FITC), One-step TUNEL In Situ Apoptosis Kit". Images of brain slices were obtained using a Nikon Eclipse 80i microscope with a Nikon DS-Fi1c color camera at a magnification of ×100 for analysis and ×200 for photographic materials at a resolution of 1280×960 pixels using the NIS-Elements AR 4.20.00 software application. Apoptotic TUNEL cells were counted on 4–5 slices of the studied area of the brain of each animal.

Statistical processing of the experimental data obtained was carried out in the MS EXCEL spreadsheet processor using the Data Analysis application software and the specialized statistical analysis software Statistica v.10. The methods of frequency, variance, factor, and correlation analysis were used. The significance of differences between the groups was assessed using the rank-sum test (White), the Fisher's exact probability test for frequency analysis, the F-criterion of the analysis-of-variance (ANOVA).

RESULTS

Observation of animals during the acute intoxication period showed that toxic effects signs (according to the sum of points characterizing the features of appearance, muscle tone, motor activity, and reaction to gripping) were detected on 1–2 days, with a rapid normalization of the condition by days 5–7 in the survived animals (Fig. 1).

The clinical picture of acute phenylcarbamate intoxication was dominated by signs of seizures and ataxia. Methanol poisoning led to a decreased muscle tone and motor activity, impaired coordination of movements and posture, decreased sensitivity and reflexes. Under lead acetate intoxication, the enteropathogenic symptom complex, irritation of the peritoneum, impaired motor activity and respiration were observed. At the same time, different toxicants were characterized by a different rate of increase and decrease in the symptoms of intoxication (the fastest and most pronounced manifestation of intoxication, an intensive decrease for phenylcarbamate, a slower decrease for methanol). Lead acetate was characterized by a slower increase in poisoning symptoms, which is associated with the peculiarities of its absorption in the body.

During dynamic observation of animals, no statistically significant differences were found between the control group and the groups of animal survivors of acute intoxication in terms of such indicators as body weight, feed intake, paw grip strength, retention time on a rotating rod, as well as in terms of individual indicators of the neurophysiological techniques used (locomotor activity, emotional lability, aggressiveness, grooming in the open field test; locomotor activity and staying in the illuminated sector during the CPAR test; indicators of reflex response to harsh sound and latency characteristics in the Startle Response System test). Therefore, these indicators were not reflected in the tables and figures following below.

On day 30 of the study, signs of impaired ingestive (feeding and drinking) behavior, reproduction of CPAR,

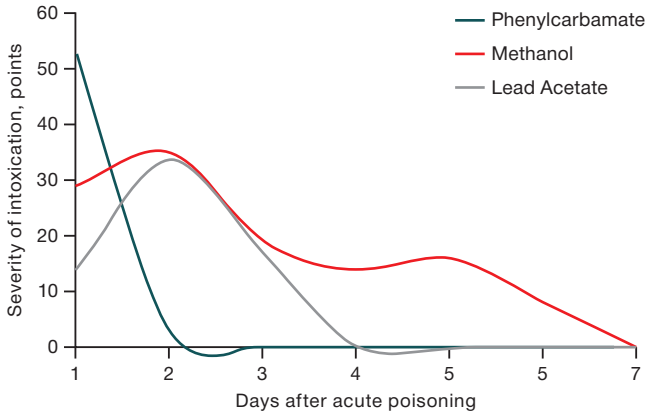


Figure prepared by the authors based on their own data

Fig. 1. Dynamics of intoxication severity after administration of LD_{50} toxicants

coordination of movements, inhibition in polysynaptic reflex circuits, depletion of activation influences were revealed (Table 1). The totality of these signs allowed the animal poisoning survivors to be divided into subgroups according to the level of formation of functional signs of the long-term health consequences of acute poisoning.

Table 1 indicates that early stages after acute intoxication (seven days) were associated with a statistically significant increase in water consumption in animals treated with phenylcarbamate and methanol. Additional water intake is explained by the need to reduce the concentration of the toxicant and accelerate its excretion from the body.

In the methanol exposure group, male rats demonstrated a decrease in search and research activity of 2.8 ± 0.7 units versus 9.2 ± 1.7 units in control animals, which is a manifestation of the direct toxic effect of the substance. The animals also showed disorders in the reproduction of conditioned reflexes in the CPAR test, a post-stress effect in animals from the phenylcarbamate group of 33 ± 13 s versus 4 ± 3 s in the control group, a shortening of the reaction time to a flash of light of 7 ± 1 ms versus 13 ± 3 ms in the control group. The group of animals exposed to methanol demonstrated a decrease in the amplitude of the reaction in response to an electrical stimulus of 46 ± 15

Table 1. Dynamics of neurophysiological parameters in the post-intoxication period

Indicator, test	Group	Days after intoxication			
		7	15	21	28
Water consumption, mL/day #	Control	26.9	25.6	27.5	28.1
	Phenylcarbamate	32.8*	32.2	33.3*	36.8*
	Methanol	32.0*	35.5*	21.1	20.0*
	Lead Acetate	24.0	23.0	21.1	20.0*
Search and research activity, units	Control	9.2 ± 1.7	8.9 ± 1.6	6.0 ± 1.5	4.9 ± 1.0
	Phenylcarbamate	11.4 ± 1.1	10.2 ± 1.2	6.2 ± 1.3	5.6 ± 1.3
	Methanol	2.8 ± 0.7*	3.1 ± 0.8*	4.5 ± 0.4	4.9 ± 1.0
	Lead Acetate	14.6 ± 0.9	12.4 ± 2.0	8.2 ± 2.0	9.2 ± 1.2*
Duration of stay in a dark cell, s	Control	29 ± 15	-	-	4 ± 3
	Phenylcarbamate	75 ± 11*	-	-	33 ± 13*
	Methanol	35 ± 14	-	-	24 ± 13
	Lead Acetate	35 ± 23	-	-	40 ± 2.5*
Treadmill, proportion of completed the test, %	Control	100	100	100	100
	Phenylcarbamate	89	100	78	33
	Methanol	100	0♦	44	44
	Lead Acetate	100	30	70	0♦
Response to sound, amplitude, units	Intact	76 ± 34	123 ± 63		247 ± 113
	Phenylcarbamate	153 ± 72	271 ± 135		303 ± 134
	Methanol	61 ± 39	248 ± 33		159 ± 59
	Lead Acetate	76 ± 31	112 ± 58		79 ± 25
Reaction to light, duration, ms	Control	13 ± 3	9 ± 2		15 ± 2
	Phenylcarbamate	14 ± 2	9 ± 4		16 ± 2
	Methanol	7 ± 1*	9 ± 1		21 ± 2*
	Lead Acetate	20 ± 5	9 ± 4		31 ± 2•
Response to an electrical stimulus, amplitude, units	Control	224 ± 46	293 ± 111		618 ± 80
	Phenylcarbamate	283 ± 67	358 ± 78		537 ± 145
	Methanol	46 ± 15♦	337 ± 60		315 ± 63♦
	Lead Acetate	202 ± 70	360 ± 140		221 ± 74♦

Table prepared by the authors based on their own data

Notes: the data is presented as the mean value and the standard error of the mean ($M \pm m$); the indicator "water consumption" is presented as the mean group value; "–" — not investigated; statistically significant differences with the control animal group: * — $p < 0.05$; ♦ — $p < 0.01$; • — $p < 0.001$.

units, compared with animals from the control group, 224 ± 46 units. These signs correspond to a prolonged period of acute intoxication pattern.

In the following periods, the severity of the above changes was mostly leveled. At the end point of the study (day 28), the animals from the PC group retained increased water consumption and impaired conditioned reflex reproduction, which had been noted in the early stages after intoxication.

In animals exposed to methanol, the initial increase in water consumption was replaced by its decrease on day 21. Along with this, the early increase in the duration of the reaction to a flash of light and a decrease in the amplitude of the response to an electrical stimulus were leveled by the end of the experiment.

In the group of animals who survived lead acetate poisoning, a decrease in water consumption was noted throughout the observation period with its increase by day 28. These animals also demonstrated disorders of conditioned reflexes reproduction, reactions to light (31 ± 2 ms versus 15 ± 2 ms in the control) and sound, with these indicators being more significant than those in the methanol-exposed group. In addition, these animals were unable to perform a treadmill running test; in the open field test, they showed some fussiness, accompanied by an increased search and research activity.

An analysis of the detected dynamics of toxic effects and neurophysiological indicators revealed signs that are sensitive to the formation of long-term consequences of acute poisoning. These include impaired ingestive behavior and its endocrine regulation (decreased water consumption), impaired conditioned reflex reproduction, increased sensitivity to stress (time spent in a dark chamber in the CPAR test), inability to perform the treadmill running test at a high speed of the tape, inhibition in the polysynaptic pathways (high reaction time light in the TSE Startle Response System test), and insufficiency (exhaustion) of activation effects (low amplitude of the response to an electrical stimulus in the TSE Starter Response System test). Altogether, these signs made it possible to obtain an integral quantitative value of the level of formation of long-term consequences based on a point-based assessment system.

Individual variations in the studied indicators of brain metabolism were noted, with the corresponding data presented in Table 2.

An analysis of the data presented in Table 2 shows a moderate decrease in S100 protein by 30% ($p < 0.05$) in survivors on day 28 after poisoning (the period of formation of long-term consequences of acute poisoning) under the influence of acute phenylcarbamate intoxication.

The group of animals exposed to methanol poisoning showed a statistically significant increase in the activity of SOD by 90% and G-6-FDG by 37%, as well as a statistical tendency to decrease the activity of S100 protein by 27% with an increase in the activity of antioxidant defense enzymes (glutathione transferase and glutathione peroxidase by 20–21%). This observation may be indicative of compensatory processes in brain tissue in the post-intoxication period.

On day 28 after acute intoxication with lead acetate, survived animals demonstrated a statistical tendency to increase the marker of neuronal damage NSE by 94% and a threefold increase in the concentration of LPO final products — DCs — with a decrease in glutathione transferase activity by 10%, reflecting the neurotoxicity of this compound. However, in comparision with other toxicants, lead acetate exposure resulted in a more pronounced, statistically significant decrease in the activity of S100 protein by 46% and MBP by 23%, an increase in the activity of the brain neurotrophic factor BDNF by 42%, as well as a moderate increase in the activity of G-6-FDG by 18%. These indicators manifest a high activity of compensatory and adaptive biochemical mechanisms during this period.

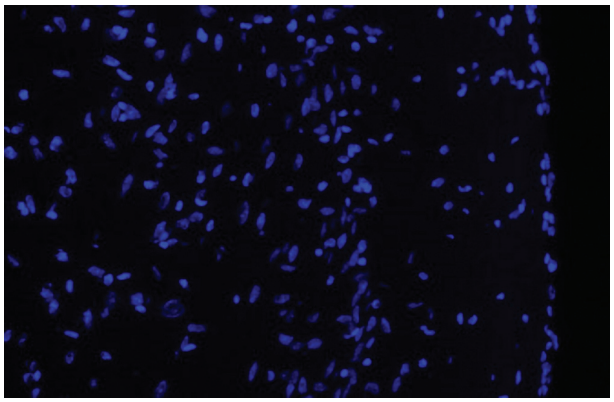
It should be noted that neurospecific proteins detected in blood serum 30 days after acute poisoning proved to be uninformative for detecting and assessing the severity of long-term health effects of intoxication. Only for lead acetate, metabolic signs of neuronal damage and, probably,

Table 2. Indicators of brain metabolism in survived animals on day 28 after acute poisoning with neurotoxicants

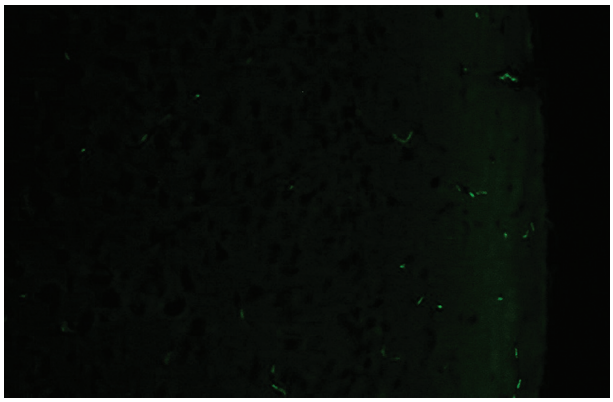
Indicator, unit	Control (n = 8)	Phenylcarbamate (n = 9)	Methanol (n = 10)	Lead Acetate (n = 5)
NSE, pg/mL	0.71 ± 0.11	0.65 ± 0.07	0.99 ± 0.17	1.38 ± 0.39*
BDNF, ng/mL	1.22 ± 0.12	1.15 ± 0.04	1.30 ± 0.09	1.73 ± 0.11*
MBP, ng/mL	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
S100, ng/mL	0.120 ± 0.011	0.084 ± 0.007*	0.087 ± 0.012	0.064 ± 0.006*
GSH, mmol/L	2.00 ± 0.05	1.95 ± 0.04	1.91 ± 0.04	1.94 ± 0.05
MDA, mmol/L	19.1 ± 1.7	19.2 ± 0.9	22.8 ± 2.3	18.9 ± 1.0
DC, mmol/L	65.3 ± 1.6	98.7 ± 36.0	126.7 ± 52.9	197.2 ± 80.6
GST, U/g of protein	62.7 ± 1.2	57.8 ± 3.5	75.2 ± 6.0	56.2 ± 1.2
GP, U/g of protein	1.45 ± 0.03	1.42 ± 0.08	1.76 ± 0.16	1.40 ± 0.04
G6PDH, U/g of protein	35.8 ± 2.3	38.2 ± 3.2	49.2 ± 5.0*	42.4 ± 2.3
SOD, U/g of protein	21.5 ± 5.3	24.2 ± 5.7	50.0 ± 5.4*	20.5 ± 5.4

Table prepared by the authors using their own data

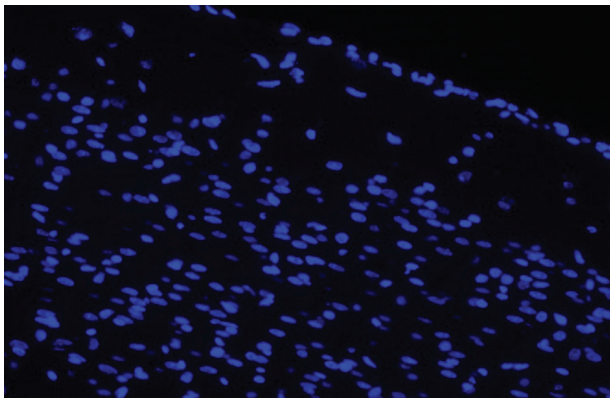
Note: the data are presented as the mean value and the standard error of the mean ($M \pm m$); statistically significant differences with the group of intact animals: * — $p < 0.05$.



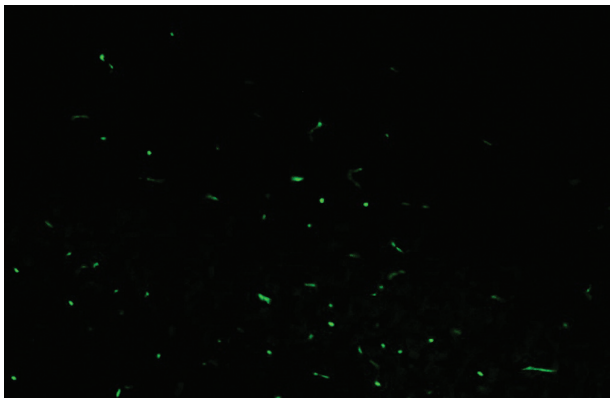
Control, DAPI — nuclear neurons in a cross section, ×200



Contol, TUNEL — apoptotic neurons, ×200



Phenylcarbamate, DAPI — nuclear neurons in a cross section, ×200



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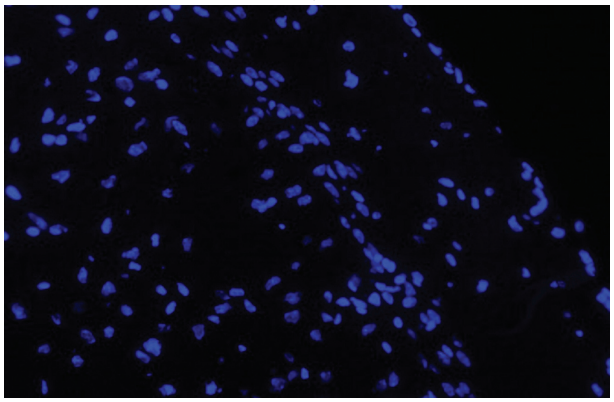
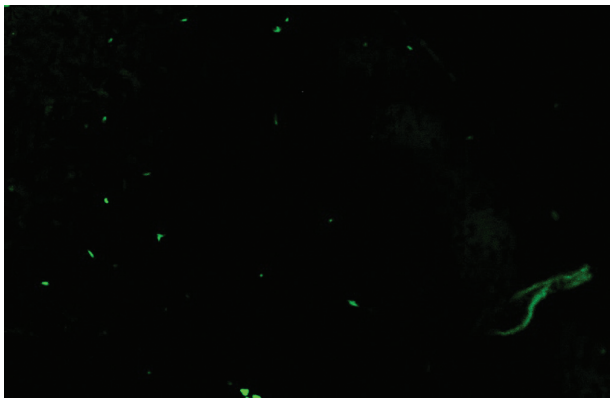
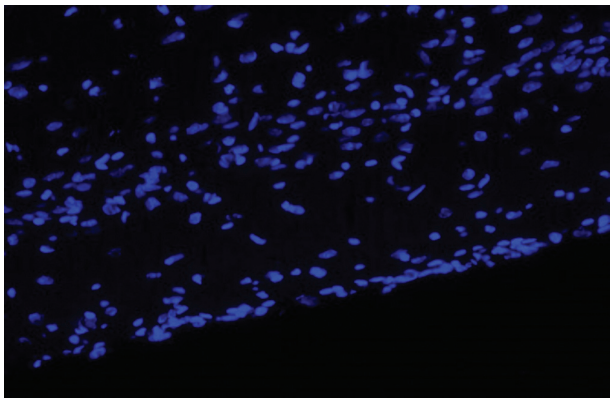


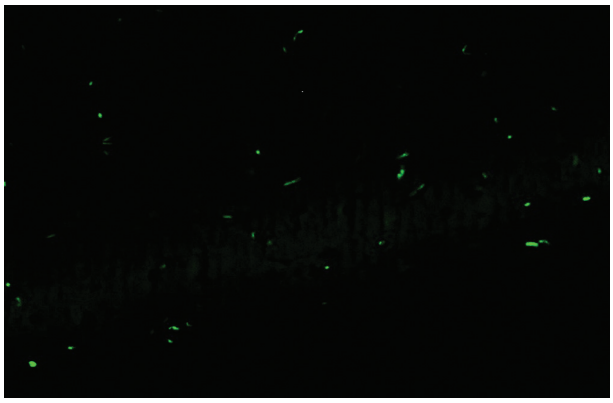
Methanol, DAPI — nuclear neurons in a cross section, ×200



Methanol, TUNEL — apoptotic neurons, ×200



Lead acetate, DAPI — nuclear neurons in a cross section, ×200



Lead acetate, TUNEL — apoptotic neurons, ×200

Figure prepared by the authors using their own data

Fig. 2. Slice micrographs of the rat brain temporal cortex

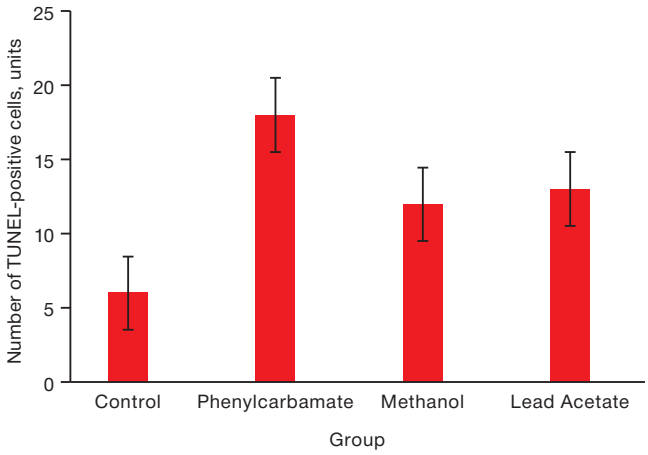


Figure prepared by the authors using their own data

Fig. 3. The activity of apoptotic processes in the cells of the rat brain temporal cortex on day 28 after acute poisoning with neurotoxicants
Note: * $p < 0.05$ — statistically significant differences from the control group

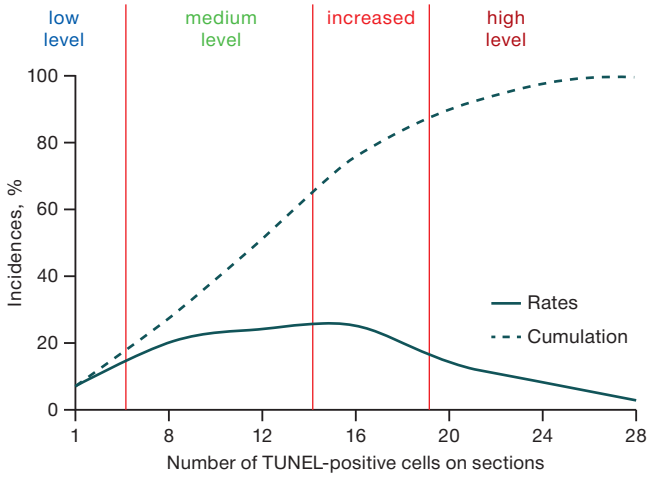


Figure prepared by the authors using their own data

Fig. 4. Frequency distribution curves of the number of TUNEL-positive cells on sections of the rat brain temporal cortex

increased permeability of the blood–brain barrier were found. It is important that lead acetate is characterized by the activation of LPO processes in the setting of a decrease in the activity of the enzymatic link of the antioxidant system. These findings are likely to be correlated with the ability known for heavy metals to inhibit the enzymatic activity of a wide range of enzymes due to binding to thiol groups in the active site.

Figure 2 demonstrates slice micrographs of the rat brain temporal cortex to assess the effect of toxicants on the processes of neuronal apoptosis.

Under almost the same number of blue-colored nuclear neurons on a slice of the cerebral cortex (left photos), the number of apoptotic TUNEL-positive nerve cells in the survived animals significantly increased under the influence of toxicants (green glow, right row of photos, Fig. 2).

Figure 3 presents the results of assessing the activity of apoptotic processes.

The data presented in Fig. 3 shows that all the studied toxicants, regardless of the mechanisms of their toxic effect, increase the number of neurons in the state of apoptosis by 3–4 times, thereby being the inducers of this pathological process.

A variance factor analysis showed that the severity of neuron apoptosis in the rat cerebral cortex is closely related to the formation of long-term consequences of severe acute poisoning with neurotropic toxicants. This indicator is associated with more than 20% of the total variability in the assessment of their formation ($R^2 = 0.47$, $p = 0.034$) in survivor animals. In this regard, the subsequent analysis was focused on assessing the significance of the influence of the studied metabolic factors on the activity level of neuronal apoptosis processes.

It follows from Fig. 3 that, on day 28, a pronounced sharp activation of apoptotic processes was observed in the brain cells of the survived animals, which is more characteristic of phenylcarbamate. The variance factor analysis showed that the controlled factor of intoxication fact determines 64% of the total variability ($p = 5\times10^{-8}$) of the apoptosis index. The type of neurotoxicant is even more important in activating apoptosis (72% of the total variation of the trait, $p = 2\times10^{-6}$), while the coefficient of determination for phenylcarbamate equaled D = 0.97 ($p = 5\times10^{-13}$), for methanol D = 0.86 ($p = 4\times10^{-8}$), for lead acetate D = 0.80 ($p = 4\times10^{-5}$).

In order to assess the level of apoptosis activation during the formation of long-term health consequences of acute poisoning, a frequency analysis and S-scaling were performed. To that end, the inflection points on the curves of the statistical distribution of the number of TUNEL-positive cells in slices were determined (Fig. 4), which are the boundaries of the levels of apoptosis activation. If the indicator under study can be characterized as close to a normal statistical distribution, such criterion points on the cumulative curve will be 16% (the boundary of low and medium levels), 66% (the boundary of medium and elevated levels), and 84% (the boundary of elevated and high levels). It is noteworthy that apoptosis induction was more pronounced (by four times) when exposed to phenylcarbamate, while the apoptosis-inducing activity of methanol and lead acetate was weaker (by about 30%).

The boundaries of the ranges (Fig. 4) are as follows: low-level — 6 or fewer TUNEL-positive cells per slice; medium-level — 7–10 cells, elevated — 14–18 cells, high-level — 19 or more cells per slice.

The following structure of apoptosis activation levels was revealed in the experimental groups (Table 3).

Table 3 shows that phenylcarbamate is a strong apoptosis inducer, causing predominantly a high level of its activation, while methanol and lead acetate cause moderate activation of programmed cell death of neurons.

The conducted analysis of the role of individual components of the brain metabolic systems in the activation of apoptotic processes under the action of neurotoxicants found that the studied metabolic parameters do not play a significant role in the process of brain cell apoptosis at the stage of formation of the long-term consequences of severe acute poisoning with neurotropic toxicants. Thus, their coefficients of determination ranged from 0.02 to 0.11, not being statistically significant. For neurospecific proteins, this may probably be due to both the features of toxic brain damage at the tissue level and the timing after acute intoxication, which triggered the activation of apoptosis.

Table 3. Distribution of animals by levels of apoptosis activation under the influence of neurotoxicants

Group	Level of apoptosis activation			
	low	moderate	increased	high
Control	88	12	–	–
Phenylcarbamate (1.6 mg/kg bw)	–	–	33	67
Methanol (11.5 g/kg bw)	–	100	–	–
Lead Acetate (300 mg/kg bw)	–	80	–	20

Table prepared by the authors using their own data

Note: the data is presented in the form of % incidence in the group.

Thus, NSE is known to reach a maximum in cerebrospinal fluid one day after an ischemic or hemorrhagic stroke, decreasing by days 4–5 and being more specific to focal brain damage than diffuse [12–15]. S100 protein also shows typical periods for increasing its concentration, i.e., days 2–7. MBP is a marker of axonal damage and demyelination, which may not have formed by the time of 30 days from the moment of acute intoxication. BDNF is more overexpressed with a lack of neuronal plasticity, and the weak response of this neurotrophin to brain tissue damage is clinically more significant [17–18].

In the group of intact animals, the apoptosis level had no significant correlations with either neurospecific proteins or LPO indicators and the antioxidant system (AOS), being a spontaneous process. In the group of animals exposed to phenylcarbamate intoxication, the apoptosis level was modestly affected by a low level of reduced glutathione (r = –0.53). After methanol intoxication, a weak correlation between apoptosis and the content of the main protein myelin (r = +0.47) was revealed, most likely reflecting the presence of a common inducing factor for them. After intoxication with lead acetate, the activity of neuronal apoptotic processes was associated with a low activity of glutathione transferase (r = –0.66) and SOD (r = –0.47). Therefore, the use of activators of these enzymes can be useful in the set of measures for preventing the long-term consequences of poisoning with organic lead derivatives.

NSE in the group of intact animals showed moderate negative associations with the activity of glutathione-dependent AOS enzymes — GST (r = –0.63) and GP (r = –0.57), i.e., an increase in the content of this marker protein may reflect the weakness of the AOS enzymatic link. At the same time, a positive correlation was found with the level of reduced glutathione (r = +0.75). Taken together, these connections may indicate a link between the damage to the bodies of neurons and the disorder of the ability to utilize reduced glutathione in antioxidant defense reactions, since the latter results in a simultaneous increase in its level and a decrease in the activity of utilization enzymes. This pattern is also observed in phenylcarbamate poisoning; however, there is an additional decrease in glucose-6-phosphate dehydrogenase activity (r = –0.67) and a positive correlation with S100 protein (r = +0.70). Following methanol intoxication, accumulation of utilized reduced glutathione (r = +0.55) is also noted; however, this is combined with lower BDNF values (r = –0.50). A peculiar pattern of NSE relationships is noted after lead acetate intoxication. An alternative activation of the expression of either NSE or S100 protein was revealed (with the correlation coefficient between them of r = –0.81). Moreover, the low activity of AOS shifts the system toward increasing the level of S100 protein. In lead intoxication, a pronounced positive correlation (almost linear) was found between the level of NSE and the activity of SOD (r = +0.94), which implies a single response to lead poisoning. After phenylcarbamate poisoning, this marker negatively correlates with the activity of glutathione-dependent AOS enzymes. In case of methanol poisoning, an increase in the amount of this protein in brain tissues is associated with the accumulation of MDA (r = +0.61) and a compensatory substrate increase in SOD activity (r = +0.57). A similar correlation between MBR and SOD was also found after lead acetate poisoning (r = +0.53).

In phenylcarbamate poisoning, the BDNF level correlated with diene conjugates (r = +0.61). After methanol poisoning, it was correlated with a deficiency of reduced glutathione (r = –0.60). After lead poisoning, the neuroprotective effects of BDNF were recorded, including a decrease in axonal damage by the S100 protein marker (r = –0.66) and an improvement in antioxidant protection (a decrease in diene conjugates (r = –0.62), an increase in the activity of glutathione transferase (r = +0.68), glutathione peroxidase (r = +0.61) and SOD (r = +0.49).

Protein S100 in the group of intact animals and in methanol poisoning showed a moderate correlation with SOD (r = +0.62). After phenylcarbamate poisoning, it correlates with the level of NSE (r = +0.70), which may reflect the process of parallel damage to both neuron bodies and axons. After poisoning with lead acetate, the level of S100 protein increases with insufficiency of the antioxidant system, i.e., low levels of reduced glutathione (r = –0.51), GST (r = –0.61), and, in particular, GP (r = –0.84) and SOD (r = –0.75).

With regard to LPO and AOS indicators, a dense cluster of positively correlating activity indicators of glutathione-dependent enzymes (GST and GP) and glucose-6-phosphate dehydrogenase was isolated in the brain tissues of intact animals (correlation coefficients in the range from +0.70 to +0.98). In case of poisoning with phenylcarbamate and methanol, SOD joins this cluster, the density of correlations decreases slightly. At the same time, after poisoning with lead acetate, this cluster disintegrates, glutathione peroxidase becomes the key antioxidant enzyme, and SOD is associated with a moderate correlation with glucose-6-phosphate dehydrogenase.

CONCLUSION

The conducted research showed that the common effects shared by the analyzed toxicants include an increase in the number of neurons dying by apoptosis (most pronounced with phenylcarbamate intoxication) and a decrease in the blood serum level of S100 protein (most pronounced with lead acetate poisoning). Moderate activation of antioxidant protection enzymes was specific for the long-term effects of methanol poisoning, likely as a compensatory reaction against the activation of LPO processes. The long-term effects of lead acetate poisoning in survivor animals were manifested by increased serum levels of NSE and BDNF, decreased protein S100 and MBP, increased levels of diene conjugates with decreased GST activity, and moderate activation of glucose-6-phosphate dehydrogenase. The latter is a key enzyme in the pentose phosphate pathway for the formation of reduced forms of coenzymes for the oxidation of energy metabolism substrates in brain tissues.

It was established that phenylcarbamate exhibits the properties of a strong inducer of apoptosis of cerebral cortex cells, while methanol and lead acetate are inducers with moderate activity.

It should be noted that the calculated coefficients of determination exhibit low or moderate values (for glutathione transferase and glutathione peroxidase). This suggests that neurospecific proteins, lipid peroxidation and antioxidant protection processes do not significantly affect the processes of neuronal apoptosis in survived animals at the stage of formation of long-term health consequences of severe acute poisoning. In this regard, future research should address the effect of metabolic processes in animal brain tissue on the activity of apoptotic processes and the formation of long-term consequences at an earlier stage after acute intoxication with neurotropic toxicants.

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