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EXPERIMENTAL ARTICLES

Blockage of the Action of the Proneurotoxin MPTP and Toxin MPP^+ by Extracts of Homogenates of *Alphitobius diaperinus* Litter Beetles in an Experimental Model of Parkinson's Disease

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Abstract—Adult male mice C57BL/6 ($n = 105$) were divided into five groups. The first group served as a control. In the 2nd–5th groups, the animals were treated subcutaneously with 40 mg/kg of proneurotoxin MPTP (methylphenyltetrahydropyridine), which forms a state similar to the initial stage of Parkinson's disease over a 2-week period. Mice of groups 3–5 daily received an additive along with their food: one of three extracts of the biomass of the litter beetle *Alphitobius diaperinus*. In 2 weeks, all animals were tested for motor disorders in the vertical bar test; they were then euthanized and histochemical analysis of the dopamine-containing brain regions was performed. In addition, the same extracts were tested for counteraction to MPP^+ toxin in cultured neuroblastoma cells. It was found that the primary aqueous and, especially, secondary water–methanol extracts had a powerful protective effect against the neurotoxic effect judging by the results of both the behavioral test and morphological control. Arginine was found at substantial concentrations in both effective extracts. An in vitro study confirmed the protective effect of the primary aqueous extract.

Keywords: extract of homogenate of litter beetle, *Alphitobius diaperinus*, experimental model of Parkinson's disease, motor dysfunction, cell culture of neuroblastoma, protective effect

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INTRODUCTION

Parkinson's disease (PD) is a chronic progressive degenerative disease of the central nervous system, which is clinically manifested, in particular, as a disturbance of coordination of movements. PD is one of the most socially important diseases; the study of its biological basis is the most important task of all of modern neuroscience. This disease is associated with very slow (for dozens of years) degeneration of the dopaminergic neurons of substantia nigra pars compacta in the midbrain (SNpc) that project to the striatum nuclei. The process proceeds asymptomatically as a result of the functioning of compensatory mechanisms, and motor and then cognitive disturbances occur only at a late stage of the disease, when less than half of the initial number of dopamine-containing neurons remain and the level of dopamine delivered to the striatum by these neurons falls by a factor of 4. However, it is too late to start treatment at this period. The history of world medicine knows of no patients who were cured. Modern medicine can only alleviate the symptoms of the disease and, in some cases, slow the development of the disease. Therefore, the creation of adequate experimental models and the search for early disease markers are primary tasks [1, 2].

Modern drug therapy of PD is compensation of the dopamine deficit, which develops in PD as a result of the death of dopaminergic neurons of the brain, as well as inhibition of disease progression (neuroprotective therapy). PD pharmacotherapy includes cholinolytic agents, aminoadamantane derivatives, DOPA-containing agents, type B monoamine oxidase inhibitors (MAO-B), catechol-O-methyltransferase inhibitors, and dopamine receptor agonists [3–5]. Despite a fairly wide range of medications and developed drugs for PD therapy, the task of finding new drugs for this purpose remains urgent, which results not only from the insufficient effectiveness of known drugs but also from their side effects [2, 6, 7].

Progress in the study of the pathogenesis of neurodegenerative diseases depends primarily on the development of adequate experimental models. The models

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of preclinical and early clinical stages of PD are of the greatest interest. The latter stage is characterized by a threshold level of degradation of the nigrostriatic dopaminergic system and the first minor disturbances of the motor function. These models provide a unique opportunity to study the pathogenesis of the disease, the molecular mechanisms of neurodegeneration and neuroplasticity, at its early stage, to search for peripheral biomarkers as a basis for creation preclinical diagnostics, and to identify new molecular targets for pharmacotherapy, primarily for neuroprotection. Obviously, neuroprotective treatment can be effective only if it is initiated at the early stage, rather than the late stage, when most of the neurons have already died [8].

One of the universally recognized PD models is a model based on the use of the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). This model was founded due to seven Californian drug addicts with marked symptoms of parkinsonism, who were discovered in 1983. It turned out that these young people practiced intravenous injections of crude meperidine, a synthetic analogue of heroin, which contained high concentrations of a by-product of synthesis, MPTP. Thorough research carried out in the following years showed that the MPTP molecule, due to its high lipophilicity, easily passes through the blood–brain barrier, penetrates into astrocytes and, under the influence of the enzyme MAO-B, is converted into the MPP^+ ion (the 1-methyl-4-phenylpyridine ion). This ion binds to the high affinity dopamine carrier protein (DAT) and thus penetrates the mitochondria of dopamine-containing neurons. It then inhibits complex-1 (associated with the enzyme NADH-ubiquinone oxidoreductase) of the mitochondrial electron-transfer chain and, thus, uncouples oxidative phosphorylation. This, in turn, leads to a disruption of ATP production, an increase in the level of extracellular calcium, the formation of free radicals/reactive oxygen species which, after interaction with cellular proteins, nucleic acids, lipids, and other molecules, cause cellular damage and, ultimately, neuronal death, i.e., manifestations of dopamine neurotoxicity [9–11].

A PD model using C57BL/6 black mice was developed, which is now universally recognized and validated by the International Society of Psychopharmacologists. In accordance with this model, mice are systemically administered with the proneurotoxin MPTP, which selectively destroys the dopaminergic system. The effect of this toxin depends on the dosage and mode of administration. It was shown that a single administration of 40 mg/kg (1/2 of LD_{50} dose) models the early clinical stage of PD. The stages were identified by the first manifestations of a motor behavior disorder, as well as by the results of a histological study [12].

Insects have long attracted the attention of researchers as a source of biologically active substances. As an example, the bee *Apis mellifera*, the larvae and beetles of the mealworm beetle *Tenebrio molitor*, the blow fly of the Calliphoridae family, the wax moth *Galleria mellonella*, and the silkworm *Bombyx mori* contain new antimicrobial peptides, chitin-melanin complexes, flavonoids, amino acids, and organic acids [13–20].

The litter beetles of the family Tenebrionidae, which are used in folk medicine to treat a wide range of diseases, are of great interest. These insects synthesize protective secretions, which are a mixture of repellent and blocking chemoreceptor substances. These are located in the cuticular inclusions or abdominal glands and are released when the beetles are stressed. It appears that the secretions of the beetles are also necessary for the insect to prevent drying out and to protect it from pathogenic microorganisms [21–24]. It was shown that these secretions are a source of pharmacologically active compounds that are promising for the treatment of respiratory diseases [25]. Current data suggest substances with anti-inflammatory and immunomodulating properties, as well as cytotoxicity with respect to the cells of some tumors, which were isolated from extracts of the litter beetle *Ulomoides dermestoides,* may be used in medicine [26–28].

The litter beetle *Alphitobius diaperinus*, which belongs to the family Tenebrionidae like *U. dermestoides,* is broadly distributed in the temperate and southern zone of the European part of Russia and in Siberia [29]. We suggested that some components of the biomass of the *A. diaperinus* beetle may have inhibitory activity against the postponed effects of the proneurotoxin MPTP, which causes experimental parkinsonism in C57BL/6JSto mice [30, 31]. Preliminary experiments with the model of the early clinical stage of PD (systemic single injection of MPTP at a dose of 40 mg/kg) showed that an *A. diaperinus* homogenate immobilized on a plant carrier possesses a pronounced ability to neutralize the postponed effect of proneurotoxin MPTP in mice when testing the physical endurance of animals on a rotating rod (Rotarode) 2 weeks after injection. Histological studies revealed a decrease in the amount of tyrosine hydroxylase (TH)-immunopositive neurons in the SNpc region of mice administered with the proneurotoxin compared to intact mice and animals that received a prophylactic treatment with the *A. diaperinus* homogenate on the plant carrier. There were no significant differences in dopaminergic neurons between intact mice and animals that received the homogenate with food. The results indicated the absence of characteristic lesions of dopaminergic neurons of SNpc in mice that received both a proneurotoxin and antidote [30].

Subsequent study showed that when extracting beetle biomass, the water–methanol extract obtained after solid-phase extraction has the greatest effect as an antidote to proneutoxin MPTP. Behavioral data

were obtained by testing animals on a vertical rode [31]. These results required further research.

The objectives of this study are as follows:

(1) verification of the working hypothesis on the ability of water–methanol extracts from homogenate of adult *A. diaperinus* beetle to counteract motor dysfunction and destruction of TH-containing SNpc neurons caused by systemic administration of the proneurotoxin MPTP in the model of early PD stage in mice in vivo;

(2) the detection of the action of extracts on the cells of SH-SY5Y neuroblastoma in the presence of the neurotoxin MPP^+, in vitro.

MATERIALS AND METHODS

The biomass of the adult beetle *A. diaperinus* was obtained by cultivation of the insect in the Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences under controlled conditions on wheat bran. Five grams of freshly harvested biomass after separation from the feed substrate was immobilized at –18°C, then homogenized, filled with chilled distilled water (1 : 3) and extracted for 24 hours in the cold. It was then centrifuged at 12000 *g* for 5 min. The fugate was passed through a 0.22 μm Millipore bacterial filter (Sigma) and 15 mL of an aqueous extract (Extract-1) was obtained; 10 mL of this extract was collected and stored at –4°C and 5 mL was further processed.

A secondary water–methanol extract was prepared by adding methanol to 5 mL of the aqueous extract-1. After separation of the precipitate by centrifugation at 12000 *g* for 5 min, the supernatant was passed through a Sep-Pack C18 Classic (Waters Corporation) cartridge and then evaporated on a rotary evaporator and dispersed in 5 mL of distilled water on a S30H ultrasound bath (Elma Schmidbauer), which yielded extract-2 . The remaining substances on the cartridge were washed away from the cartridge with methanol and the filtrate was evaporated on a rotary evaporator and dispersed in 5 mL of distilled water in an ultrasonic bath (extract-3).

The composition of the extracts was studied by mass spectrometry on an LCQ Advantage Max (Thermo Scientific) device with electrospray ionization, direct syringe injection of samples in the form of methanol solutions with an extract concentration of 10 μL/mL.

The arginine concentration was measured by HPLC as dansyl derivatives prepared according to a modified method [32]. Beta-alanine (Sigma) was used as an internal standard. For a reaction, 10 μL of the extract to be analyzed, 5 μg of Beta-alanine solution in water, 70 μL 50 mM Li_2CO_3, 30 μL acetonitrile, and 10 μL of a solution of dansyl chloride in acetonitrile (10 mg/mL) were added to an Eppendorf centrifugal microtube (1.5 mL).The reaction time was 3 hours at room temperature, in the dark. After the reaction, 10 μL of glacial acetic acid was added to the tube for neutralization.

HPLC was performed on a MiliChrom-A02 chromatograph (EkoNova). A Prontosil 120-5-C18aq column was used, 2 × 80 mm. Eluent: A = 0.2 M $LiClO_4$ + 5 mM $HClO_4$; B is methanol; the gradient was piecewise linear; 0 min, 15% B; 8.5 min, 50% B; 10 min, 50% B; 16.5 min, 100% B; 20 min, 100% B. The flow rate was 150 μL/min. Detection was at 250 nm. The volume of the test sample was 3 μL.

The extracts were immobilized on sterile edible wheat bran; the final moisture content of the mass was 8%. Preparations were stored in the refrigerator and fed to the experimental animals daily for a week, for which the dry preparations were added to the main fodder for mice by fractional and thorough mixing (8 g of the preparation per 1 kg of feed mix). The main feed mix consisted of porridge, including boiled oats and peas, with the addition of vegetable oil. The consumption of the studied extracts by mice was standardized by dispensing the feed in an amount corresponding to the daily consumption rate for mice (11 grams of thermally processed cereals).

Five groups were composed of mice with similar weights (young mature males of the C57BL/6JSto strain, $n = 104$):

group 1, control (animals were injected with physiological saline instead of toxin and feed mixture was given without additives, $n = 27$);

group 2, toxin without extract (mice were injected with toxin and feed mixture was given without additives, $n = 25$);

group 3, toxin + extract-1 (mice were injected with toxin and a feed mix was prepared with the addition of extract-1 (primary aqueous extract, $n = 21$);

group 4, toxin + extract-2 (mice were injected with toxin and a feed mix was prepared with the addition of extract-2 (secondary aqueous methanol extract, $n = 21$);

group 5, toxin + extract-3 (mice were injected with toxin and feed mixture was added with the addition of extract-3 (residue, washed from the Sep-Pack C18 cartridge, $n = 10$).

The scheme of the experiment included the following stages:

(1) preliminary preventive maintenance of experimental mice for 1 week on a ration with preparations;

(2) administration of proneurotoxin MPTP to the animals and continuation of feeding with drugs for 2 weeks;

(3) detection of the first signs of Parkinson's disease using the vertical bar behavioral test;

(4) histochemical analysis of the presence of brain lesions;

(5) determination of the possibility of using the culture of neuroblastoma cells SH-SY5Y to evaluate

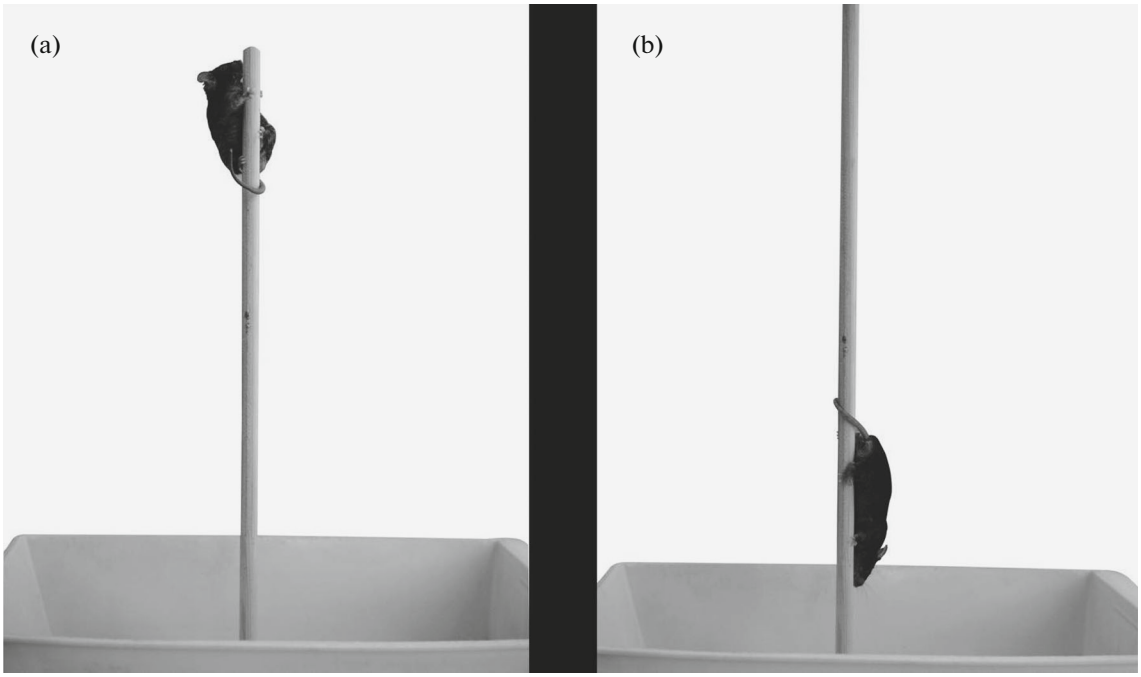

Fig. 1. The vertical bar behavioral test. (a) The initial position of the mouse; (b) descent.

the properties of *A. diaperinus* beetle extracts to inhibit the cytotoxic effect of MPP^+.

During the first week of the experiment, mice from the groups 3–5 received the appropriate preparations in the feed mix. Animals of the groups 1 and 2 received a similar diet without drugs. Subsequently, proneurotoxin MPTP (Sigma, United States) was administered once subcutaneously to animals in groups 2 to 5, at a dose of 40 mg/kg, after which the mice received the same food for the next 2 weeks as before injection. Mice of the control group 1 received the main feed mixture without drugs during the entire experiment; these mice were injected with saline instead of toxin.

Two weeks after administration of the toxin, the presence of sensorimotor disorders in the vertical bar test [33–38] in our modification was determined in mice of all groups [31, 39]. Individual testing was performed in a home cage. At the beginning of the test, a vertical bar with an unpolished rough surface, 50 cm high and 1 cm in diameter was placed in the cage with the mouse. Close to the top of the bar, a mouse was placed on it so that its head was oriented vertically upwards. Once on the pole, the mouse turned the position of its body with its head vertically down and started to descend from the bar to the bottom of the cell (Fig. 1). Using a stopwatch, the time of descent to the bottom of the cage, which was covered with sawdust, was recorded. Even if the animal after reorientation descended but not to the end of the bar, and jumped off at some distance from the cage bottom, we recorded the time spent by mouse to reach the cage bottom. In our version of the technique, three tests were performed with every mouse. The minimum time interval between tests was 1 min. The test was considered as passed if the mouse managed to reorient its body and began descent during the first 150 seconds of testing. If the mouse did not descend from the bar during the first 150 s in two or all three trials, its data were not taken into account in the statistical analysis. For statistical analysis, the maximum time for descent from the bar was selected for every mouse (in two or three trials), since this indicator allows one to estimate locomotor dysfunctions in mice with clinical signs of experimentally induced parkinsonism. The results of the experiments were processed using Unistat 6.5.04 software via the Kruskal–Wallis rank test, while the Conover–Inman test was used for pairwise a posteriori multiple comparisons.

The brain damage of mice during PD development, which is associated with the destruction of dopamine-containing neurons, was evaluated by counting dopaminergic neurons immunohistochemically stained for a key enzyme of catecholamine biosynthesis: tyrosine hydroxylase (TH). The animals were euthanized by intraperitoneal injection of a lethal dose of urethane (over 1 g/kg). The blood system was transcardially perfused with a solution of phosphate buffered saline PBS (Sigma, United States), pH 7.4, and then with a 4% formaldehyde solution in PBS. After additional fixation in a formaldehyde solution for 12 hours at +4°C, the brain was impregnated with a 30% sucrose solution in PBS for 24 hours at +4°C. Subsequently, the brain was separated by a freezing microtome (MSE, United Kingdom). Frontal 40-μm

sections containing the substantia nigra were collected in PBS. For immunohistochemical staining, each fourth section was placed in a solution of monoclonal mouse anti-TH antibodies (T2928, Sigma, United States) diluted 1 : 200 in PBS solution supplemented with 2% normal horse serum, and 0.3% Triton X-100 detergent (Sigma). The sections were kept at 4–8°C in this solution for 12 hours with constant stirring. After washing in PBS, the sections were immersed for 1 hour in a solution of biotinylated anti-mouse immunoglobulin antibodies (Vector Laboratories, United States) diluted 1 : 100 in PBS and 0.3% Triton X-100 at room temperature. After three washes in PBS, the slices were placed in a solution of the ABC complex (Vector Laboratories) in PBS at a dilution of 1 : 200 also for 1 hour, and a standard peroxidase reaction was performed with 0.03% diaminobenzidine (Sigma) solution in PBS supplemented with 0.01% hydrogen peroxide. The colored sections were placed on slides and covered with 50% glycerol and a cover slip. Quantitative analysis of TH-positive cells on immunohistochemically stained sections was performed using an Olympus IX81 microscope (Olympus, Japan) equipped with a Marzhauser motorized stage (Marzhauser Wetzlar, Germany) controlled by a computer and an Olympus DP72 digital camera. The cells were counted on a computer monitor using the Cell program (Olympus Soft Imaging Solution, Germany) using the optical fractionator method [40].

To detect the effect of *A. diaperinus* extracts on SH-SY5Y cells, neuroblastoma cells were cultured for 48 hours in the presence of the MPP^+ toxin (1 mM and 2 mM) and various extracts of the litter beetle. The number of live cells after the action of the MPP^+ toxin at a given concentration was taken as 100% of the viability. Cell proliferation was measured by co-administration of the extract with 0.1 or 0.2 mM dopaminergic MPP^+ toxin (methylphenylpyridine ion, Sigma) into RPMI culture medium containing 10% fcs. The cell proliferation efficiency was assessed using the MTT test. This test is based on the ability of mitochondrial dehydrogenases of live cells to convert the yellow MTT (3-(4,5-dimethylthiazolyl-2)2,5-diphenyl tetrazolium bromide) (PanEco) into blue formazan that is insoluble in aqueous solutions [41]. Cells were plated in 96-well plates (5000 per well) in 90 μL of standard culture medium. After 20 hours of incubation, 10 μL of the extract and toxin were added to the cells. The final concentration of the extract in the culture solution was 1%, while the toxin was 1 mM or 2 mM. Cells were incubated for another 48 hours, after which 20 μL MTT (5 μg/mL diluted in physiological saline solution) was added to each well for 3 hours. After 3 hours of incubation, an enzymatic reaction occurred in live cells and formazan was generated, which accumulated as crystals in the cells. The solution was then carefully removed from the wells and 60 μL DMSO (dimethylsulfoxide) (Khimmed) was added to each well to dissolve the formazan crystals and shaken thoroughly until completely dissolution. The quantitative determination of formazan was performed on a multichannel photometer (Thermo scientific) with a 530 nm filter. Cell viability was assessed according to the ratio of the optical density in the control wells (without the addition of substances) and in the wells with the substance.

RESULTS

Three extracts were obtained from the biomass of the adult *A. diaperinus* beetle: a primary aqueous extract (extract-1), a secondary water–methanol extract (extract-2) and a wash extract from the Sep-Pack C18 cartridge (extract-3). Mass spectrometry of the extracts revealed the presence of arginine as the dominant component in the spectrogram (Fig. 2, upper spectrum). The fragmentation spectrum of the arginine peak 175@35 eV coincides with the library spectrum for arginine (Fig. 2, average spectrum), which identifies this substance as L-arginine. The quantitative determination of the arginine content in the extracts by HPLC as dansyl derivatives yielded results in the range from 10 to 17 mg/mL extract (Fig. 2, lower spectrum). The concentration of arginine in extract 1 and extract 2 was the same (arginine was not lost in the preparation of the methanol extract). Extract 3 did not contain arginine.

Experimental dry formulations were prepared on the basis of the corresponding three extracts, after which three groups of experimental C57BL/6JSto mice were fed for 3 weeks. At 1 week after the initiation of prophylactic feeding with drugs, an injection of proneurotoxin MPTP was made according to the scheme described in the methods. After the injection, 15% of the mice died within the first 24 hours from stress and acute toxicity. No deaths of the surviving animals during the subsequent experiment were observed. After completion of the experimental feeding, we analyzed the maximum time of descent from the vertical bar in all mice.

Figure 3 shows the maximum descent time of the mice. Table 1 shows the data on the Conover–Inman test (Q) statistics and the significance of the differences in the descent time between the experimental groups. It may be seen that pairwise a posteriori comparisons using the Conover–Inman test revealed a significant increase in the maximum descent time in the mice of the group 2 (toxin without extract) in comparison with group 1 (control, $Q = 3.9696$, $p = 0.0002$), group 3 (toxin + extract-1, $Q = 2.1351$, $p = 0.0358$), and group 4 (toxin + extract-2, $Q = 5.0300$, $p < 0.0001$).

The pairwise comparisons of the experimental groups 2 and 5 ($Q = 1.4681$, $p = 0.1459$), 3 and 1 ($Q = 1.9569$, $p = 0.0538$), 1 and 4 ($Q = 1.3183$, $p = 0.1911$) did not reveal significant differences in the maximum duration of descent from the bar. In group 3, the max-

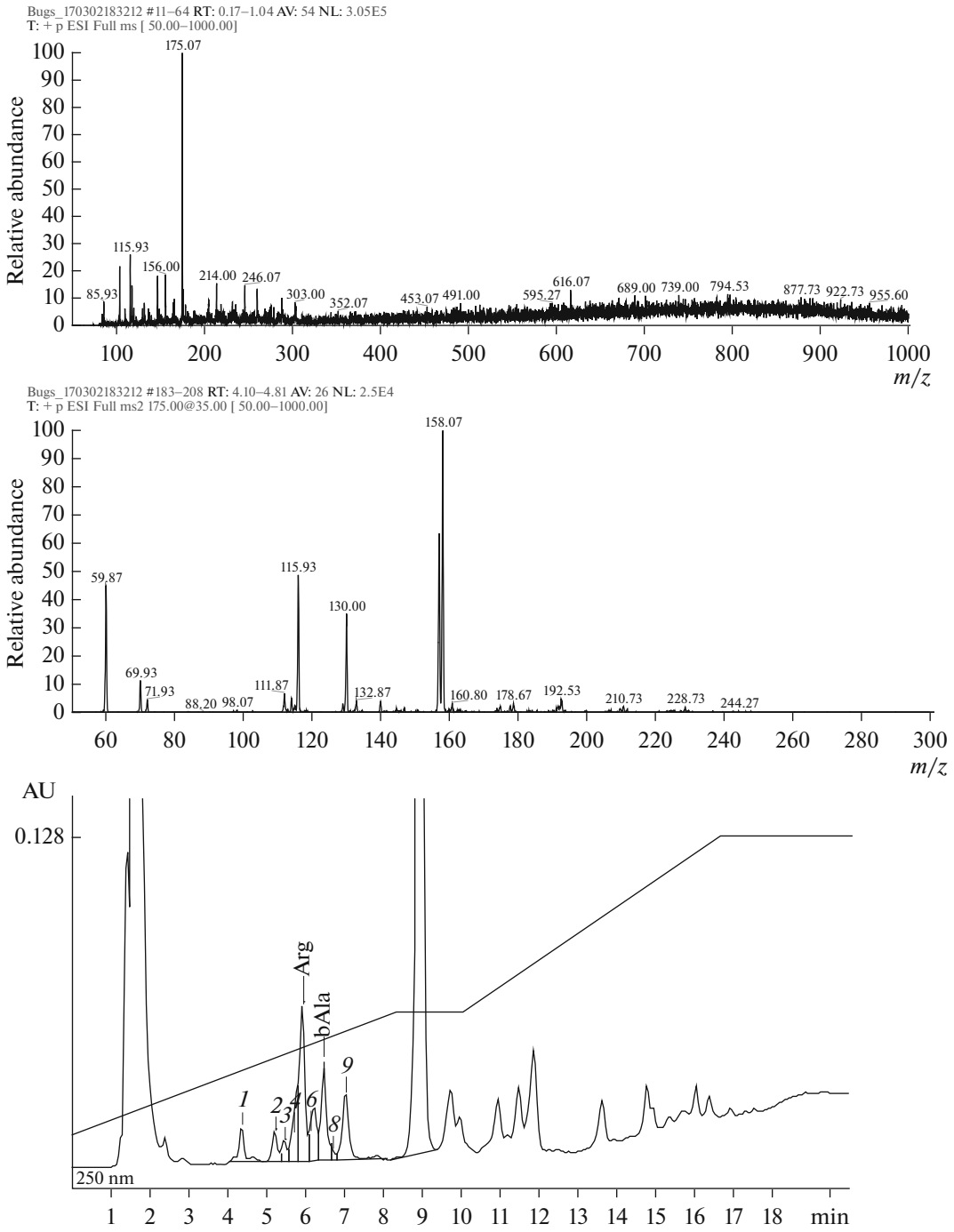

Fig. 2. Top panel, mass spectrum of extract-1, 1 μL/mL in methanol. Peak 175 is arginine. In the middle is the mass spectrum of fragmentation of the molecular peak of arginine 175@35 eV. Below is the determination of the arginine concentration by the HPLC method in the form of dansyl derivatives. Sample: extract-1, 10 μL. The concentration of arginine in the extract is 16.3 mg/mL.

imum duration of descent from the bar was significantly greater than in group 4 ($Q = 3.1712$, $p = 0.0021$). The results shown in Fig. 3 show the statistical distribution of the data.

Thus, the administration of the proneurotoxin MPTP significantly and reliably reduced the motor coordination of mice in the vertical rod test, which was manifested in the increased maximum descent time of animals of group 2 (44.8 ± 7.5 s) compared to the control group 1 (16.7 ± 2.8 s). Both extracts, aqueous (group 3) and secondary water–methanol (group 4) extracts, protected against the proneurotoxin,

smoothing the effects of MPTP and reducing the time of descent from the vertical bar (the time of descent of the mice was 28.7 ± 5.6 s in mice of group 3 and 12.2 ± 1.2 s in the group 4, respectively). The secondary water–methanol extract (group 4) appeared to be not only more effective in neutralizing the action of proneurotoxin MPTP (group 2) than the primary aqueous extract (group 3) but even had a stimulatory effect on the animals. The coordination and motor activity of group 4 mice were higher than the control mice of group 1 by 4.6 s.

After the completion of the experimental feeding of animals, a histochemical study of the brain was performed. Table 2 shows the total number of TH-containing neurons in the substantia nigra pars compacta and the ventral tegmental area (mean and distribution) in all five groups of mice. Figure 4 shows an example of the frontal sections of the midbrain from mice from the first, second, and third groups, as the most contrasting ones. The administration of extracts of the *A. diaperinus* beetle into the diet of mice according to the prophylactic scheme reduced the toxic effect of the MPTP and allowed the preservation of dopamine-containing neurons in animals from group 3 (toxin + extract-1) and 4 (toxin + extract-2) completely, and partially from group 5 (toxin + extract-3).

The results of an in vitro study of the effects of *A. diaperinus* extracts on SH-SY5Y cells in the presence of MPP^+ toxin are shown in Fig. 5. The number of living cells after the combined exposure to the toxin at two concentrations and the antidote in the form of three extracts is normalized to the effect of the toxin itself (without the antidote), which was taken as 100%. It may be seen that the primary aqueous extract-1 reduced the toxic effect of MPP^+ at 2 mM concentration by 25%, and the secondary water–methanol extract-2 reduced the same toxic effect by 13%. This was manifested in a corresponding increase in the number of viable cells. The residue washed from the Sep-Pack C18 cartridge (extract-3) had no protective effect.

DISCUSSION

Using a behavioral test in experiments on C57BL/6JSto mice, we obtained data on the protective properties of an aqueous and secondary water–methanol extract of the *A. diaperinus* beetle against the proneurotoxin MPTP and on the prophylactics of the development of the experimental model of Parkinson's disease by food that contained the extracts.

The primary aqueous extract-1 contained components, whose isolation by the method of solid-phase extraction SPE [42] made it possible to obtain the more efficient secondary water–methanol extract-2 from the primary aqueous extract. Extract-3, which contained residual substances after secondary water–methanol extraction, had no protective effect and the

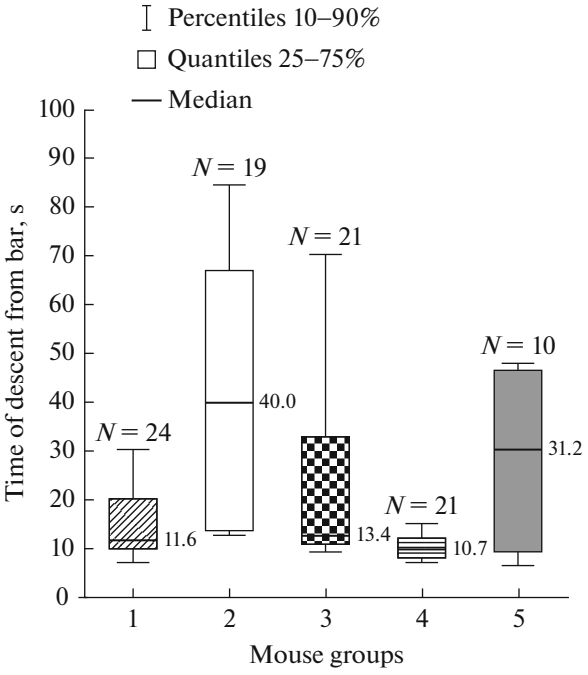

Fig. 3. The median of the maximum (in three trials) time of mouse descent of from the bar (in seconds) in the experimental groups.

time of descent of mice from the vertical bar was not significantly different from the analogous index of animals from group 2 (toxin without extract). The primary aqueous extract showed a large statistical distribution of the data, which may be due to some instability of the extract, since it may contain enzymes that lyse the active substances or oxidizers that inactivate them. The secondary water–methanol extract-2 demonstrated a more stable effect on mice. This suggests that the substance (substances) in the secondary

Table 1. The statistics of the Conover–Inman test (Q) and the significance of the differences in the time of descent from the bar between the experimental groups of mice (* $p \le 0.05$; ** $p \le 0.01$)

Compared groups	Q statistics	Significance of differences (p)
1 and 2	3.9696	0.0002**
1 and 3	1.9569	0.0538
1 and 4	1.3183	0.1911
1 and 5	1.1156	0.2679
2 and 3	2.1351	0.0358*
2 and 4	5.0300	0.0000**
2 and 5	1.4681	0.1459
3 and 4	3.1712	0.0021**
3 and 5	0.0730	0.9420
4 and 5	1.8937	0.0618

Table 2. The results of the histochemical analysis of the brain

Index	Group of mice				
	1 control	2 toxin without extract	3 toxin + extract-1	4 toxin + extract-2	5 toxin + extract-3
Number of TH-containing neurons in SNpc + VTA (M ± SEM)	11797 ± 703*	3435 ± 308	13472 ± 610*	15360 ± 823*	8064 ± 464

*As compared to group 2, U-test, $p < 0.05$.

water–methanol extract-2 contributed to the effective compensation of clinical manifestations of the initial stage of the experimentally induced PD in C57 mice and improved brain activity.

The effect of proneurotoxin MPTP in mice was also expressed in a significant (by 70%) reduction in the number of dopamine-containing neurons in the SNpc region of the brain in animals from group 2 (toxin without extract) compared to controls (group 1), which corresponds to the literature data [12, 30].

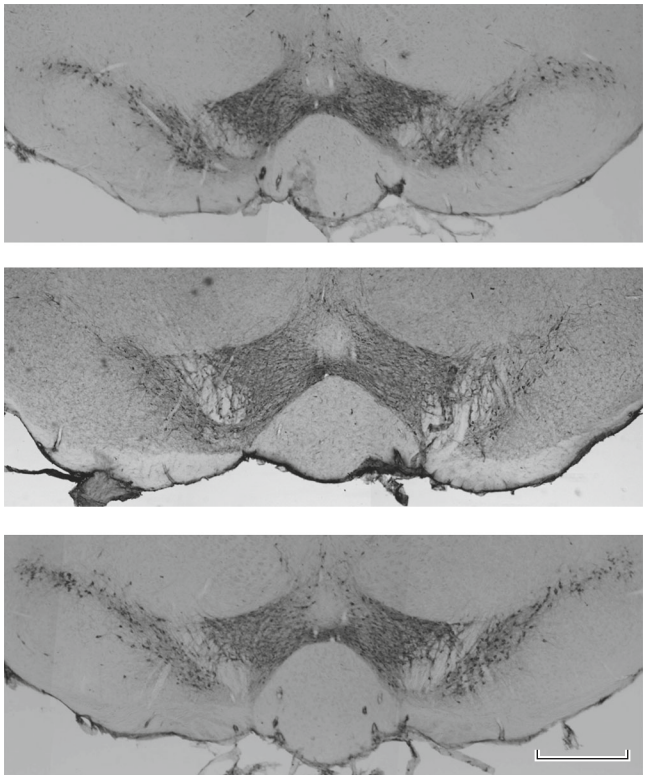

Fig. 4. A transverse section of the brain. Top panel, the mouse from the control group 1. The cells containing tyrosine hydroxylase are dark colored. The center of image, the area of the ventral tegmental area; diverging wings are substantia nigra pars compacta. The middle panel, the same area in the mouse brain from group 2 (toxin). Bottom panel, the same region in the mouse brain from group 3 (toxin + extract-1) 2 weeks after administration of subcutaneously proneurotoxin MPTP. The scale is 1 mm.

These results generally correspond to the indices of the behavioral test of motor coordination on the vertical bar. Given that physiologically active extracts of the beetle entered the animals with food, and that the effect was found at the level of the morphology of the brain cells, it may be assumed that low-molecular water-soluble compounds that enter the bloodstream through the circulatory system and reach the brain are active, penetrate the blood–brain barrier, and protect dopaminergic neurons.

This effect may develop via at least two paths. First, through the inhibition of the glial MAO-B enzyme, which transforms the propreneurotoxin MPTP into the neurotoxin MPP^+ and, secondly, through the activation of the glial neurotrophic factor, which is a protective protein of dopaminergic neurons [43].

The observed presence of L-arginine in the extracts may indicate that this amino acid is an important component of the extracts that protects against Parkinson's disease. It is known that arginine, which is a donor of nitric oxide, the most potent of the endogenous vasodilators, is also a precursor of ADMA (asymmetric dimethylarginine), an endogenous inhibitor of NO synthase. Thus, this is a mechanism of

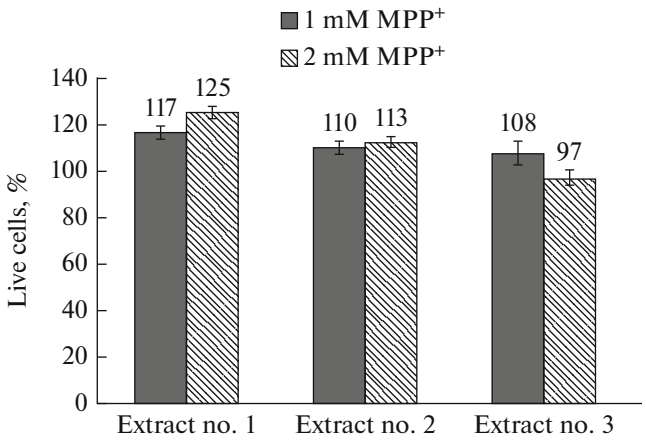

Fig. 5. The protective effect of *A. diaperinus* extracts on the SH-SY5Y cells. The number of living cells (along the Y-axis) after the combined exposure of the toxin at concentrations of 1 and 2 mM and the antidote in the form of three extracts was normalized to the effect of the toxin itself (without the antidote) taken as 100%.

the self-regulation of NO production in the body. In PD, especially in the late stages of the disease, the arginine/ADMA ratio is disturbed, which leads to NO insufficiency. It thus appears that ADMA is a risk factor for PD and can participate in its pathogenesis. Modern therapy of PD includes consumption of arginine as a food supplement along with vitamins B6 and B12 and folates [7, 44, 45].

The in vitro effects in the culture of SH-SY5Y neuroblastoma cells only partially correlated with the results we obtained in intact animals in vivo. Thus, the use of the SH-SY5Y cell culture as a model for studying the interaction of MPP^+ toxin with various fractions of *A. diaperinus* beetle extracts requires further study. Nevertheless, the basic antitoxic effect of extracts from *A. diaperinus* beetle have been shown in cell culture. The causes of the lower protective effectiveness of the secondary water–methanol extract compared to the primary aqueous extract on the culture of neuroblastoma cells that do not correspond to the behavioral response of mice may be explained by the presence of a broader spectrum of substances in the primary aqueous extract than in the secondary aqueous methanol extract. These substances, which remained in the Sep-Pack C18 cartridge after passing in the water–methanol extract, enhance the effect of the extract-2 components on the cells. In addition, the mice were given dry preparations and the cell culture was treated with extracts. The active substances that act against the PD in the primary aqueous extract were probably destroyed in the preparative form, which reduced the biological effectiveness of the preparation. The preparative form of the secondary water–methanol extract was more stable during storage; thus, it produced a greater effect.

CONCLUSIONS

Here it was shown for the first time that the pronounced sensorimotor disturbances in mice 2 weeks after systemic administration of 40 mg/kg of proneurotoxin MPTP, which were revealed in the vertical bar test, were reduced or absent if the animals received an antidote in the form of primary aqueous or secondary water–methanol extracts of the biomass of *A. diaperinus* beetle in their food. The results of the histochemical analysis of the number of dopamine-containing neurons in the brain showed the preservation of pigmented neurons after toxin injection when the animals were kept on a preventive diet with aqueous extracts of the beetle biomass homogenate and corresponded to the data obtained in the behavioral test with a vertical bar. The culture of the SH-SY5Y neuroblastoma cells reacted to a lesser extent to the action of the extracts; however, we found a definite protective action of the primary aqueous extract of the litter beetle and, to a lesser extent, of the secondary water–methanol extract.

CONFLICT OF INTEREST

The authors declared no conflicts of interest.

ETHICAL APPROVAL

All experiments were performed in accordance with the humanitarian principles of the directives of the European Community (2010/63/EU).

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