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Cerebroprotective and Regenerative Effects of Alkaloid Z77 under Conditions of Brain Ischemia

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We studied the psychopharmacological effects of atisine-type diterpene alkaloid Z77 in a rat model of cerebral ischemia. Pronounced cerebroprotective effect was found consisting in normalization of the orienting and exploratory activity and conditioned behavior associated with significant correction of morphological changes in the brain. The direct stimulatory effect of Z77 on neural stem cells was shown *in vitro*.

Key Words: *regenerative medicine; cerebroprotective drugs; alkaloids; neural stem cells; cerebral ischemia*

Pharmacological action of existing cerebroprotective drugs consists mainly in the protection or modulation of functions of mature cellular elements of the nervous tissue preserved under pathological conditions [1,2]. However, this concept of pharmacological intervention is untenable in some cases. Available drugs are often unable to not only fully restore morphology and function of the brain, but also prevent the progression of neuropathology [2]. In this regard, the development of fundamentally new and pathogenetically substantiated approaches to the treatment of diseases of the CNS and creation of original cerebroprotective drugs with completely different mechanisms of action are an important problem.

The search for approaches to solving this problem is carried out within the framework of regenerative medicine. In recent decades, rapid development of cellular technology made a breakthrough in understanding of the biology of stem cells (SC) and led to the development of cell therapy, a new trend in the treatment of many diseases [1,6-8,13,14]. In this case, pharmacological stimulation of the functions of endogenous SC by imitating the activity of natural regulatory systems is the most physiological and promising approach to the solving problems of regenerative medicine including the treatment of CNS disorders.

Experiments performed at E. D. Goldberg Research Institute of Pharmacology in the course of developing "Pharmacological Strategies of Regenerative Medicine" [3,10,15] showed principal possibility and high efficiency of this approach to treatment of various diseases including pathological conditions of the CNS. Alkaloids were identified possessing a stimulating effect on the function of progenitor cells of different classes [10-12]. The mechanisms of action of these substances are underlain by the direct effect on the receptor apparatus of progenitor cells and their effect on tissue microenvironment elements [3,4,10-12]. In addition, we have identified atisine-type diterpene alkaloid Z77 and revealed its marked activity against mesenchymal progenitor elements associated with exposure to fibroblast growth factor receptors (FGFR). The above pleiotropic immediate-early growth factor is known to be essential in determining the proliferative and differentiation status also of neural SC [3,4]. Thus, the study of the therapeutic properties of alkaloid Z77 in diseases of the nervous system is of undoubted interest.

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Here we studied cerebroprotective effects of Z77 in experimental cerebral ischemia and the effect of the alkaloid on the implementation of the growth potential of neural SC.

MATERIALS AND METHODS

The work was carried out on outbred male rats (n=80) weighing 250-300 g and male CBA/CaLac mice (n=12) weighing 22-24 g. First-category conventional rats and mice were obtained from the Laboratory of Experimental Biomodels, E. D. Goldberg Research Institute of Pharmacology.

Z77 alkaloid was isolated from aboveground parts of *Ranunculaceae* plants. The substance was sequentially extracted with chloroform, sulfuric acid, ether, and chloroform and analyzed by chromatography on deactivated alumina in hexane–acetone solvent system (90→50%). The substance belonging to atisine-type diterpene alkaloids was identified and dissolved in distilled water to a final concentration of 0.00025%.

Experimental ischemia [9] was simulated in rats in the morning under ether anesthesia by complete ligation of the left carotid artery and 50% restriction of blood flow in the right carotid artery by partial ligation of the artery under the guidance of an electromagnetic flow-meter (MFV-1100, Nihon Kohden). The control group included sham-operated animals (isolation of carotid arteries without ligation).

The solution of the alkaloid Z77 was administered to rats with brain ischemia and sham-operated animals orally by gavage in a dose of 25 mg/kg once a day during 5 days.

The therapeutic effects of the substance were evaluated by functional and morphological methods. On day 3, 1 h after Z77 administration, orienting and exploratory behavior was assessed in the open-field test during the first minute and two successive minutes separately, and passive avoidance response (PAR) was conditioned [1,15]. In this case, functional asymmetry was evaluated semi-quantitatively by the width of the palpebral fissure on the side of complete ligation of the carotid artery as follows: 0 points, the left palpebral fissure is completely closed; 1 point, the left palpebral fissure is less by $^1/_3$ in comparison with the right one; 2 points, the left palpebral fissure is less by $^2/_3$ in comparison with the right one; 3 points, the left palpebral fissure is approximately closed; 4 points, the left palpebral fissure is completely closed. On day 7, PAR performance was checked in the animals, after which histological preparations of the brain were examined (fixed in 10% neutral formalin, dehydrated in an ascending alcohols, embedded in paraffin, cut into 4- to 5-μ sections, and stained with hematoxylin and eosin).

The direct impact of the alkaloid Z77 on the implementation of growth potential of neural SC was studied by cloning *in vitro* [2]. For this purpose, 5 ng/ml of the alkaloid Z77 was introduced into a liquid culture medium containing 10^5 cells/ml from the mouse paraventricular region. The culture was incubated in CO_2-incubator at 37°C, 5% CO_2 and 100% humidity for 7 days. The number of neurospheres and neural CFU (CFU-N) were counted after incubation [1,15].

The obtained data were processed by the methods of variation statistics using Student's t test and nonparametric Mann–Whitney U test.

TABLE 1. Indicators of Orienting-Exploratory Behavior in Rats in the Open-Field Test (arb. units; $X \pm m$)

Group	Total locomotor activity	Horizontal activity	Vertical activity	Hole sniffing	Hole-board test	Grooming	Defecation
At the first period of observation (1 min)							
Sham-operated	17.0±2.1$^+$	8.2±1.8$^+$	3.3±0.8	2.00±0.37	1.0±0.4	0	2.5±0.9
Ischemia	26.0±2.2	16.3±1.7	5.8±1.1	1.50±0.22	1.2±0.1	0.2±0.2	1.0±0.6
Ischemia+Z77	12.8±2.6$^+$	7.2±2.8$^+$	2.2±0.9	0.80±0.37	1.0±0.4	0	1.6±0.9
Sham-operated+Z77	16.2±1.9$^+$	10.6±2.5	2.6±0.7	0.67±0.21	0.8±0.5	0	1.3±0.7
At the second period of observation (2-3 min)							
Sham-operated	9.2±2.4	2.3±1.4	2.6±0.9	2.5±0.8	1.2±0.5	0.5±0.3	0
Ischemia	30.2±4.2	15.7±2.1	6.8±1.0	3.0±0.4	2.8±0.7	1.2±0.6	0.6±0.6
Ischemia+Z77	15.8±3.6$^+$	9.8±2.5$^+$	2.2±0.7	1.6±0.7	1.8±0.8	0.4±0.4	0.6±0.2
Sham-operated+Z77	12.6±3.0	6.3±2.8	2.8±1.0	0.3±0.2	1.3±0.4	0.8±0.4	1.0±0.5

Note. Here and in Table 2: $^+p<0.05$ in comparison with ischemia group.

RESULTS

Sesquialteral ligation of the carotid arteries resulted in a sharp asymmetry of palpebral fissures (up to1.6±0.3 points) and marked changes in the psychoneurological status. There was an increase in horizontal activity in the open field more pronounced at the second period of observation (2-3 min) (Table 1). This indicated predominantly damaged cognitive functions, but not nonspecific activation of the exploratory behavior [5]. In addition, marked deterioration in PAR formation and reproducibility was observed: percentage of animals with preserved PAR was 30% from the controls (Table 2). Histological assay of the ischemic brain showed significant hyperemia of the pia mater on the side with greater flow restriction, the narrowing of most cerebral vessels of various severity, perivascular and pericellular edema. We have found neurons with hyperchromatic nuclei with vacuolar degeneration surrounded by phagocytes, in a state of phagocytosis, and neurons with pyknotic nucleus and shrunken cytoplasm in the hippocampus (Fig. 1). Thus, sesquialteral ligation of the carotid arteries was accompanied by the development of severe ischemia causing acute structural and functional brain injury with later recovery [9]. In this case, sham-operated rats did not show any regular changes (Tables 1, 2).

Z77 administrated after simulation of the pathological condition almost completely abolished the signs of CNS disorders showing reduction of asymmetry in palpebral fissures (up to 0.3±0.1 points) and diminished total locomotor activity (mainly due to the decrease in the horizontal movements). We observed an increase in the number of animals with preserved PAR along with increase in its latency period and time spent in the dark compartment (Tables 1, 2).

The correction of examined behavioral and functional parameters was based on the restoration of the brain morphology. Alkaloid Z77 induced more uniform vascular blood filling in the right and left hemispheres, significantly reduced the severity of perivascular and pericellular edema of the nervous tissue, and lowered the number of neurons with pyknotic nucleus, vacuolar degeneration and neurons in a state of phagocytosis in the hippocampus (Fig. 1).

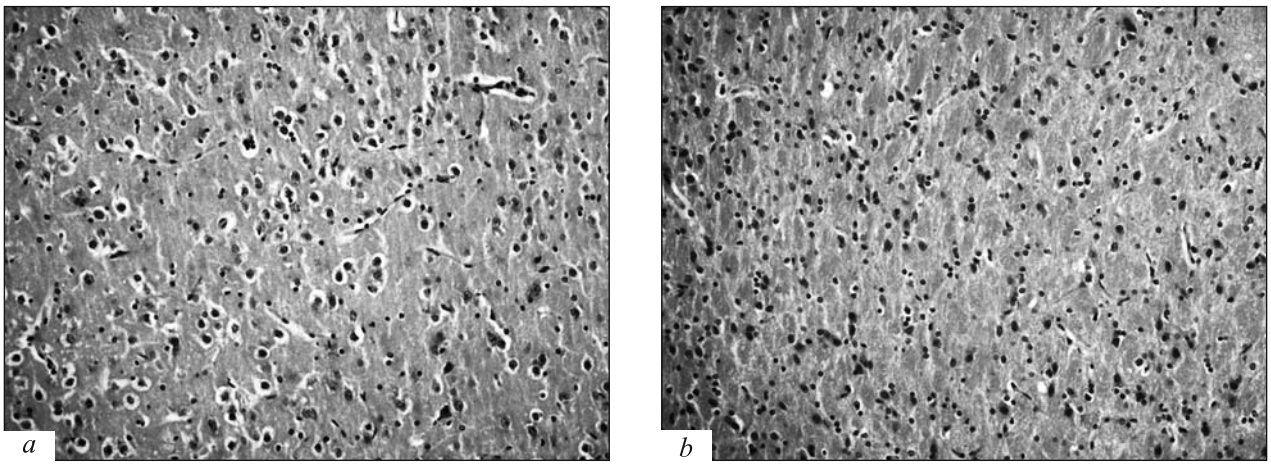

Fig. 1. Rat brain after ischemia simulation (*a*), after the introduction of the alkaloid Z77 along with ischemia simulation (*b*). Staining with hematoxylin and eosin, ×100.

TABLE 2. Indicators of Elaboration and Display of PAR in Rats (*X*±*m*)

Group	Latency of entry into the dark chamber during the elaboration of PAR, sec	Latent period of reflex response, sec	Test	
			total time staying in the dark chamber, sec	% of animal with formed PAR
Sham-operated	13.8±7.8	170.0±4.0	9.7±2.7^{+}	90^{+}
Ischemia	12.6±3.3	69.6±13.3	110±26.4	30
Ischemia+Z77	17.2±7.1	154±5.3^{+}	25.5±5.3^{+}	80^{+}
Sham-operated+Z77	11.3±2.7	149.8±17.9^{+}	30.5±12.6	80^{+}

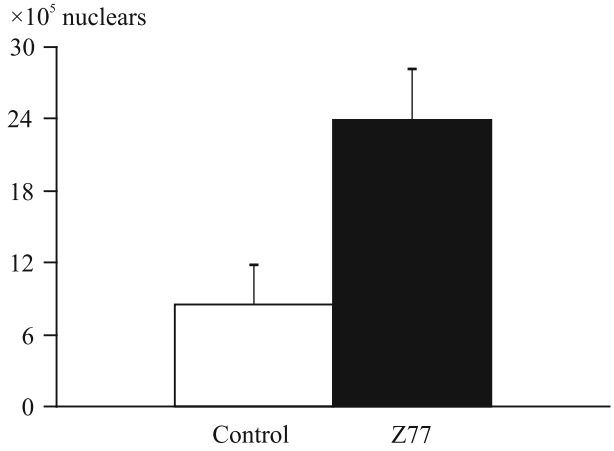

Fig. 2. Number of CFU-N in culture of intact cells from paraventricular region of the brain of male CBA/CaLac mice (controls) and after addling alkaloid Z77 to the medium. Confidence intervals at p=0.05.

The direct stimulatory impact of Z77 on neural SC was found during the study of the mechanisms of development of psychopharmacological effects. A sharp increase in the yield of neurospheres (CFU-N) after addition of this alkaloid to the culture medium (up to 278.9% from the level of intact cells; Fig. 2) was recorded *in vitro* indicating significant increase in the degree of realization of the growth potential of neural SC, probably associated with the activation of FGFR [1,4].

The results indicate the expressed cerebroprotective action of diterpene alkaloid Z77 in a cerebral ischemia model. In this, regenerative activity of the compound determined by its effect on neural SC [1,4,15] apparently had an important role in the process of restoration of the structure and functions of the CNS. However, the relatively rapid emergence of therapeutic effects indicated the anti-ischemic effect of substance, which could be due to a change in the functioning of elements of the microenvironment of nervous tissue, glial cells with membrane expression of FGFR, which is a pleiotropic and multifunctional factor [1,7,10].

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