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BIOPHYSICS
OF COMPLEX SYSTEMS

Content of Nitrogen Monoxide and Copper in the Hippocampus of a Rat Model of Short-Term Cerebral Ischemia Followed by Reperfusion

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Abstract—Electron paramagnetic resonance (EPR) spectroscopy is used to determine the content of nitric oxide (NO) and copper in the hippocampus of healthy rats and rats under ischemia modeling. Ischemia is modeled by both carotid artery ligation and carotid artery ligation, followed by taking 3 mL of blood from the common carotid artery. Signals from $(DEDTC)_2$-Fe^{2+}-NO and $Cu(DEDTC)_2$ complexes are recorded by EPR spectroscopy. A significant decrease in NO production in the hippocampus (on average 28% per day) is found after an ischemic stroke caused by carotid artery ligation; and by 56% after carotid artery ligation followed by 3 mL of blood taken from the common carotid artery. The copper content in the hippocampus the day after ischemia caused by carotid artery ligation significantly decreases by an average of 20%; after carotid artery ligation with blood sampling, there is a tendency for the copper content to decrease; however, due to the large scatter of values, the reliability of the changes cannot be confirmed. Thus, cerebral hypoxia caused by carotid artery ligation is accompanied by a decrease in NO production in the hippocampus and signs of weakening of the antioxidant system, which further harmed the functional state of the homeostasis system.

Keywords: electron paramagnetic resonance, spin trap, nitric oxide, copper, hippocampus, ischemia, hypoxia
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INTRODUCTION

Nitrogen monoxide (NO) is one of the key signaling molecules that regulate the physiological functions of the body, including the nervous system [1–3]. Studies of the role of NO in the vital activity of organisms began shortly after the discovery of the regulation of NO of the normal vascular tone as a mediator of vasodilation [4, 5]. Since NO is a chemically highly reactive free radical capable of acting both as an oxidizer and as a reducing agent [6, 7], the assumption arises about its diverse effects in biological tissues. It has been demonstrated that NO participates in various functions of the nervous system, is involved in neuromodulation processes, performs the function of a neurotransmitter, and regulates the proliferation and differentiation of nerve cells [3, 8]. It has been shown that the functioning of the NO system is violated by hypoxia and cerebral ischemia; cerebral ischemia is accompanied by multiple and multidirectional changes in the NO content in the brain and in signal transmission [9–11]. Thus, clarifying the patterns of changes in the NO content in the brain during ischemic processes is important. The available contradictory information suggests that there is currently no consensus on the role of endogenous NO in the processes occurring in the damage to the nervous system [12].

One of the reasons for this situation is the variety of sources of NO. NO, the smallest known signaling molecule, is produced by three isoforms of NO synthase (NOS). Neuronal NOS (nNOS) is constitutively expressed in central and peripheral neurons, endothelial NOS (eNOS) is mainly expressed in endothelial cells, and inducible NOS (iNOS) can be expressed in many cell types. All of them use L-arginine and molecular oxygen as substrates and require several cofactors; and all NOS bind calmodulin and contain heme [13]. Not only NOS but also nitrite reductase systems are involved in the formation of NO, which are associated with heme-containing proteins capable of reducing nitrites to NO in the deoxy form [14, 15]. It has also been shown that an exogenous low molecular mass dinitrosyl iron complex with thiol-containing ligands has a wide range of biological activity that

mimics the activity of endogenous NO [16]. It has been proved that the functioning of NO in humans and animals is ensured by its inclusion in dinitrosyl iron complexes, which are the "working forms" of endogenous NO responsible for its functioning as a universal regulator of basic metabolic processes [17, 18].

Another reason for the contradictory results is the technical difficulty of determining NO levels, since NO is formed during rapid chemical reactions involving a wide range of molecules and intermediaries, including metals, thiols, free radicals, amino acids, calcium, and oxygen. There are many methods for measuring NO production in biological systems, but a precise assessment of both the stationary concentration of NO and the rate of its generation in biological systems is a difficult task due to the low activity of nitric oxide synthase and the short half-life of NO [19]. One of the most effective methods for detecting and quantifying NO in biological tissues is the electron paramagnetic resonance (EPR) [20, 21]. This is achieved with a technique developed by Professor A.F. Vanin and his colleagues [20, 22], in which the spin trap is used.

It is important to note that NO, when interacting with a superoxide (O_2^-), forms a strong oxidizing peroxynitrite ($ONOO^-$) [9]. The main cellular defense against superoxide and peroxynitrite is a group of oxidoreductases known as superoxide dismutases, which catalyze the cleavage of O_2^- into oxygen and H_2O_2 [23]. The dismutation of superoxide by the cytosolic enzyme Cu,Zn-superoxide dismutase is the primary and main protection against free radical oxidation processes.

The authors of this study have attempted to detail some biophysical patterns of nitrogen monoxide formation in cerebral ischemia. The aim of this paper is to study the intensity of NO production and the copper content (as an indicator of superoxide dismutase) by EPR spectroscopy using spin traps in the hippocampus of rats under the experimental ischemic brain damage.

MATERIALS AND METHODS

Modeling of Ischemic Stroke in Rats

The animals were kept in the standard vivarium conditions (with the maintenance of a 12/12-hour rhythm of lighting and darkness, air temperature at 23.0°C, and a stable supply and exhaust ventilation) with free access to water and food (*ad libitum*) and the same diet in accordance with the standards of keeping laboratory animals. The experiments were carried out during daylight hours on four-week-old male white rats (initial mass 139–145 g). The animals were divided into three groups (all groups of ten individuals each). The first group (Control) were intact rats that were not exposed to any influence. The second group (Ischemia-1) were anesthetized rats that were subjected to a 10 min violation of blood flow by carefully separating the vagus nerve from the carotid arteries and ligating with silk thread 4.0 of both carotid arteries at the level of the vocal cords. The third group (Ischemia-2) were rats subjected to a 10 min violation of blood flow by similarly separating the vagus nerves from the carotid arteries and subsequent ligation of both carotid arteries at the level of the vocal cords and taking 3 mL of blood from the left common carotid artery 2 min later (rostral ligation) [24–26]. The following experimental protocol was applied: in the Control group, the rats were in a cage in the preoperative room and were not exposed to any influence. In the second group (Ischemia-1), both carotid arteries were ligated at the level of the vocal cords under anesthesia by applying ligatures for 10 min and obturation of the common carotid arteries was completed by removing the ligatures 10 min after the blood flow blockade. In the third group (Ischemia-2), as in the Ischemia-1 group, both carotid arteries were ligated and 3 mL of blood was taken 2 min after the beginning of obturation from the left common carotid artery. All surgical procedures were performed on anesthetized animals (55.6 mg/kg ketamine, 5.5 mg/kg xylazine, and 1.1 mg/kg acepromazine, intraperitoneally) [26, 27]. After 24 h from the onset of ischemia (ischemia-reperfusion), spin trap components were injected (see below). There was no mortality in rats after these operations.

Preparation of Samples for Measuring EPR Spectra

When preparing samples for measuring EPR spectra, the authors relied on the spin trap technique proposed by Professor Vanin and his colleagues [20] using the Fe^{2+} complex with diethyldithiocarbamate, $(DEDTC)_2$-Fe^{2+} [28, 29]. DEDTC-Na was administered intraperitoneally at a dose of 500 mg/kg in 2.5 mL of water [30]. A mixture of solutions of iron sulfate ($FeSO_4 \cdot 7H_2O$, Sigma, United States) at a dose of 37.5 mg /kg and sodium citrate at a dose of 187.5 mg/kg (in a volume of 1 mL of water per 300 g of animal mass), prepared immediately before administration, was injected subcutaneously into three points, the right and left thigh and into the rostral part of the interscapular region. Iron citrate is formed in a mixture of iron sulfate and sodium citrate. DEDTC-Na and iron citrate are distributed throughout the body and, when interacting, form a water-insoluble $DEDTC_2$-Fe^{2+} complex [20]. The spin trap complex with NO is characterized by an easily recognizable EPR spectrum with a *g*-factor value of 2.038 and a triplet hyperfine structure. In addition, the spin trap interacts with Cu to form the $Cu(DEDTC)_2$ complex, which can also be detected by EPR spectroscopy [31].

Hippocampal tissues were collected 30 min after the introduction of the spin trap components (one sample of about 100 mg). The selected areas were immediately frozen at the liquid nitrogen temperature and transported from Minsk to Kazan in plastic con-

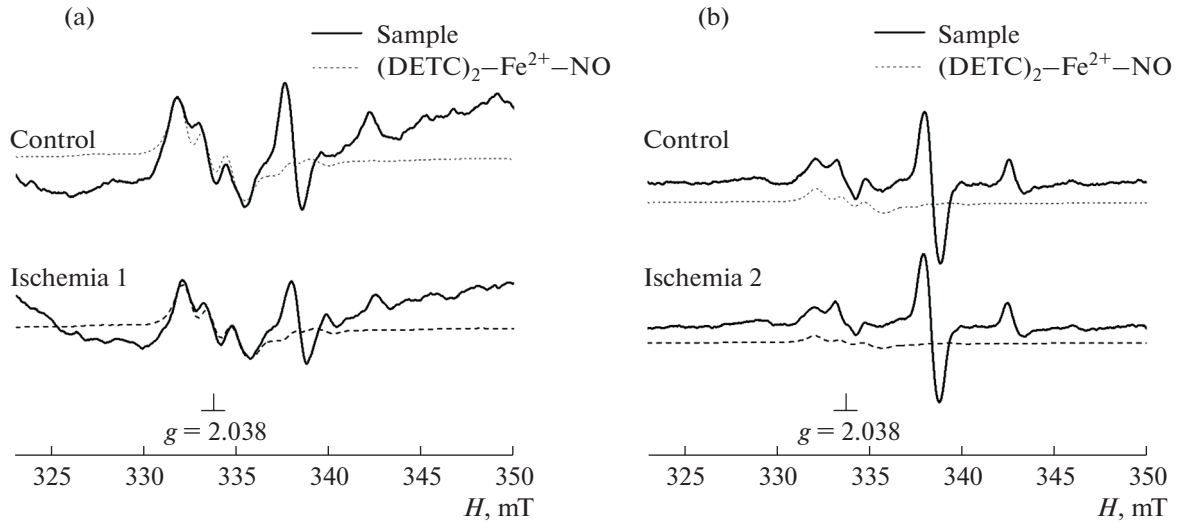

Fig. 1. Examples of EPR spectra of the hippocampus of control rats, rats subjected to a 10 min violation of blood flow by ligation of both carotid arteries (Ischemia-1), and rats subjected to a 10 min violation of blood flow by ligation of both carotid arteries, followed by taking 3 mL of blood from the common carotid artery (Ischemia-2).

tainers with dry ice for measurements by EPR spectroscopy. The spin trap complex with NO ($(DEDTC)_2$-Fe^{2+}–NO) is well preserved in this state, and the signal from the complex does not change for at least a month.

Measurements of EPR Spectra

The spectra of the $(DEDTC)_2$-Fe^{2+}–NO and $Cu(DEDTC)_2$ complexes were measured on EMX/plus Brooker X-band spectrometers (9.50 GHz) with an ER 4112HV and ER 200 SRC temperature module; the magnetic field modulation was 100 kHz, the modulation amplitude was 2 Gs, the microwave radiation power was 30 mW, the time constant was 200 ms, and the temperature was 77K in a Brooker finger Dewar vessel. The modulation amplitude, gain, and microwave power in all experiments were selected with the condition that there was no remodulation and the saturation of the EPR signal and they remained the same throughout all measurements. The mass of the samples was about 100 mg. The amplitude of the EPR spectra was always normalized by the mass of the sample (the details of the EPR signal measurements were described earlier [32]).

Statistical Processing of the Result

The results were presented as $M \pm m$ (the average value $\pm$ the standard error of the average). Statistical data processing was performed using Student's t-test. The differences were considered significant at $p < 0.05$.

RESULTS

EPR spectroscopy was used to study the intensity of nitrogen monoxide production and copper content in the hippocampus under modeling incomplete global ischemia with ligation of two carotid arteries for 10 min in rats of two groups (Ischemia-1 and Ischemia-2). In the Ischemia-1 group, cerebral ischemia was modeled by ligating two carotid arteries for 10 min, and in the Ischemia-2 group, ischemia was modeled in a reperfusion model (in addition to obturation of the common carotid arteries, 3 mL of blood was taken from the common carotid artery after 10 min) for a duration of 1 day after incomplete global ischemia with ligation of two carotid arteries for 10 min. The data obtained in the groups Ischemia-1 and Ischemia-2 were compared with the data of the Control group of rats that were not exposed to hypoxic effects and were on the standard diet in a vivarium.

Figure 1a shows the EPR spectra of the hippocampus of a control rat and a rat after modeling ischemia caused by ligation of both carotid arteries for 10 min (Ischemia-1 group). The characteristic triplet signal from the complex $(DEDTC)_2$-Fe^{2+}–NO is visible in this spectrum with a g-factor value of 2.038 [28]. In addition, a signal from the $Cu(DEDTC)_2$ complex is present in the same area. Figure 1b shows the EPR spectra of hippocampal tissues of a healthy (Control) rat and rats the day after modeling ischemia caused by ligation of the carotid arteries, followed by taking 3 mL of blood from the common carotid artery (Ischemia-2). The solid line represents the spectrum of the sample, and the intermittent line is a signal from nitric oxide associated with a spin trap, as part of the spectrum of the $(DEDTC)_2$-Fe^{2+}–NO complex. The relative change in the amount of the NO-containing complex and the $Cu(DEDTC)_2$ complex was evaluated by the integral intensity of the signal from these complexes.

Figure 2a provides statistical data on the change in the integral intensity of signals $(DEDTC)_2$-Fe^{2+}–NO

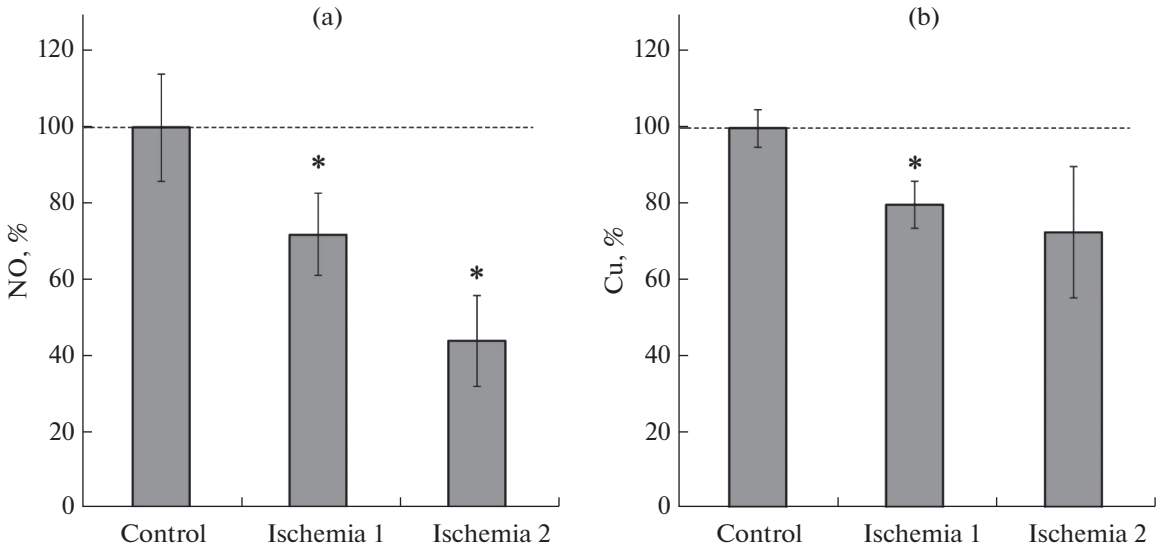

Fig. 2. The relative content of NO (a) and copper (b) (in % relative to the control) in the hippocampus of rats subjected to a 10 min violation of blood flow by ligation of both carotid arteries (Ischemia-1) and rats subjected to a 10 min violation of blood flow by ligation of both carotid arteries, followed by taking 3 mL of blood from the common carotid arteries (Ischemia-2); * $p < 0.05$.

in the spectra of the studied samples of biological tissues. The results of the analysis demonstrate a significant ($p < 0.05$) decrease in NO production after modeling ischemia in the hippocampus (Ischemia-1) caused by carotid artery ligation (by an average of 28%), and a more pronounced decrease in NO production (by an average of 56%) during carotid artery ligation followed by removal from the common carotid artery 3 mL of blood (Ischemia-2). Figure 2b provides statistical data on the integral intensities of the $Cu(DEDTC)_2$ signal in the spectra of the studied samples. The results show that the copper content in the hippocampus in animals of the second group significantly ($p < 0.05$) decreased after a day on average by 20%, and in the third group there was also a decrease in copper content; however, due to the large scatter of the values, the change was statistically unreliable. Thus, cerebral ischemia caused by carotid artery ligation was accompanied by a decrease in NO production in the hippocampus, as well as signs of weakening of the antioxidant system of the hippocampus, which together further harmed the functional state of the nervous system.

DISCUSSION

Brain stroke is the leading cause of death and the most common cause of disability worldwide [33, 34]. It is known that hypoxia is accompanied by an impaired oxygen supply to parts of the brain, which leads to cerebral ischemia and often ends with an ischemic stroke. On the one hand, the development of cerebral ischemia and the subsequent occurrence of stroke are associated with a weakening of the cerebral blood flow and with the impaired regulation of the blood supply to brain tissues by the NO system [10, 35]. On the other hand, hypoxia itself, which arises as a result of ischemic stroke, is accompanied by damage to brain tissues and impaired brain functions [36]. A weakening of the oxygen supply to the brain also occurs when a vessel is thrombosed or an aneurysm ruptures, which often results in an ischemic or hemorrhagic stroke [37, 38]. According to the research results, the role of NO in these processes of hypoxia and ischemia seems contradictory: NO is able to perform both neurotoxic and neuroprotective functions [12, 39–41].

We used EPR spectroscopy to study the intensity of nitrogen monoxide production and the copper content (as an indicator of the first and third subunits of superoxide dismutase) in the hippocampus of rats after modeling cerebral ischemia. The method developed by Professor Vanin and his colleagues [20, 28] was used; it is based on a spin trap, a water-insoluble $(DEDTC)_2$-Fe^{2+} complex, which is able to interact with NO to form a stable $(DEDTC)_2$-Fe^{2+}–NO radical detected by EPR spectroscopy. The typical EPR spectra of frozen samples (in our case, the hippocampus) have an EPR signal with $g_\perp = 2.038$, $g_\parallel = 2.01$, and a triplet hyperfine structure at $g_\perp$. The spectrum corresponds to the typical NO radical complex with a DEDTC trap. This is indicated by the value of the *g*-factor of the EPR signal and splitting due to interaction with the nitrogen nucleus. The value of the *g*-factor and the constants of the hyperfine interaction with nitrogen are in accordance with the data obtained in the work of Vanin et al. [20]. Previously, it was found that the spin trap interacts with Cu to form the $Cu(DEDTC)_2$ complex, which is also determined by EPR spectroscopy [31]. The presence of a signal from

copper was demonstrated in our measurements. It was shown that the copper content associated with the superoxide dismutase content in the hippocampus either decreased or tended to decrease a day after ischemia modeling. This result indicated a decrease in the effectiveness of the antioxidant system in these models.

The results of measuring the content of nitrogen monoxide and copper obtained in this work demonstrated that brain hypoxia was accompanied by a decrease in NO production in the hippocampus, which depended on the severity of exposure. At the same time, a significant decrease in the copper content or a downward trend was recorded. This result suggested that the hypoxia modeled was accompanied not only by a decrease in NO production but also by signs of weakening of the antioxidant system of the hippocampus, which further harmed the functional state of the system.

Oxidative stress and inflammation play a crucial role in a ischemia/reperfusion injury of the brain. Oxidative stress is caused by reactive oxygen species during cerebral ischemia and is more likely to lead to cell death and, ultimately, brain damage after reperfusion [42]. Activation of antioxidant enzymes is one of the ways to protect against highly toxic oxygen radicals. Most of them are associated with copper-containing enzymes [43, 44]. These are primarily Cu, Zn superoxide dismutase, and cytochrome *c* oxidase [44–46]. Cytochrome *c* oxidase is an enzyme of the respiratory electron transfer chain that catalyzes the transfer of electrons from cytochrome *c* to oxygen. The dismutation of superoxide (O_2^-) by the cytosolic enzyme superoxide dismutase is the primary and main protection against free radical oxidation processes [23]. It plays a crucial role in the antioxidant protection of almost all cells that are in contact with oxygen in one way or another. Thus, the balance of copper in the nervous system is necessary for its normal functioning.

The role of NO in the development of ischemia has long attracted the attention of researchers. When the activity of NO synthases (NOS) was measured, it was shown that 10 min after the onset of cerebral ischemia, an increase in the activity of neuronal NOS was observed [47], followed by the start of the expression of inducible NOS [48], whose selective blocking might be a neuroprotective factor in ischemia [11]. A similar result of activation of NO production in the first few minutes was obtained by EPR spectroscopy [49]. The doubling of NO production in the hemispheres of the rat brain was also shown in the global ischemia model [46]. The neuroprotective properties of NO donors were revealed after short-term and permanent ischemic damage [39, 51–53]. We previously found a decrease in the NO content in another model after modeling an ischemic stroke in the ischemic part of the left hemisphere cortex of rats [32].

Hypoxic or ischemic stress causes many serious brain injuries, including stroke and neonatal ischemic encephalopathy. During the processes of hypoxia and cerebral ischemia, NO performs either a neurotoxic or neuroprotective role, depending on factors such as the isoform of NOS, the type of cells that produce NO, and the time stage after the onset of hypoxic-ischemic brain damage [35, 38, 54, 55]. NO has a dual identity, including neuroprotection and neurotoxicity during ischemia-reperfusion. Excessive NO production can be neurotoxic, leading to cascading excitotoxicity reactions, inflammation, apoptosis, and a deterioration of the primary brain injury. In contrast, NO, produced by endothelial NOS, plays a neuroprotective role, supporting the cerebral blood flow and preventing damage to neurons, as well as inhibiting platelet and leukocyte adhesion. Sometimes, induced NO-derived NOS and neuronal NOS in certain areas of the brain can also play a neuroprotective role [34, 55].

There are many reasons for such a variety of NO functions. Firstly, in addition to synthesis by NO synthases as the main source of NO [3, 10], there is also a nitroreductase component of the NO cycle, when NO is formed from nitrites and nitrates [14, 38]. Secondly, there are a significant number of depots for NO that interact with complexes containing iron (for example, heme structures), with thiols, and with other compounds [27, 56, 57]. The significant role of NO in many processes, including in the activity of the nervous system, as well as the lack of information about the amount of synthesized NO and its functions in various pathologies, determines the importance of further research in this direction. Therefore, solving the issue of the dynamics of NO production in tissues during the development of various processes, including pathological ones, is relevant and may contribute to the development of new approaches for the pharmacological correction of emerging disorders in the brain tissue.

CONCLUSIONS

Signals from $(DEDTC)_2$-Fe^{2+}–NO and $(DEDTC)_2$-Cu triple complexes were recorded by EPR spectroscopy. The advantage of using this method is its high degree of sensitivity due to the use of spin traps, which allows measurements of low concentrations of the compounds to be determined. By direct EPR spectroscopy measurements, a significant decrease in NO production in the hippocampus by 28% was shown a day after modeling ischemia by carotid artery ligation and by 56% in the case of carotid artery ligation followed with taking 3 mL of blood from the common carotid artery. The copper content in the hippocampus the day after modeling ischemia by carotid artery ligation reliably decreased by 20%, but the decrease in the copper content a day after carotid artery ligation with blood sampling showed a lack of reliability with a larger range of values. It is logical to conclude that the use of the EPR spectroscopy method made it possible to determine

not only the change in nitric oxide production in the hippocampus during the modeling of cerebral ischemia but also to identify signs of weakening of the antioxidant system in the same tissues in terms of the copper content as an indicator of the superoxide dismutase content.

ABBREVIATIONS AND NOTATION

NOS	NO synthase
EPR	electron paramagnetic resonance
DEDTC	diethyldithiocarbamate

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All applicable international, national and/or institutional principles of animal care and use have been followed. Experiments using laboratory animals were carried out in accordance with the norms of animal treatment regulated by the European Convention for the Protection of Vertebrates Used for Research and Scientific Purposes. Modeling of ischemia and ischemia-reperfusion was carried out at the Institute of Physiology of the National Academy of Sciences of Belarus (Minsk) in accordance with the approved protocol of the Ethics Commission of the Institute.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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