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Application of EPR Spectroscopy to Study the Content of NO and Copper in the Frontal Lobes, Hippocampus, and Liver of Rats after Cerebral Ischemia

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Abstract—To record the content of nitric oxide (NO) and copper in the brain tissues (frontal lobes and hippocampus) and liver of healthy rats and rats after ischemia modeling, the method of electron paramagnetic resonance (EPR) spectroscopy was used. Modeling of ischemia was carried out by ligation of the carotid arteries, followed by taking 3 mL of blood from the common carotid artery. Signals from the ternary complexes $(DETC)_2–Fe^{2+}–NO$ and the $Cu(DETC)_2$ complex were recorded by EPR spectroscopy. Based on direct measurements by EPR spectroscopy, it was shown that 1 day after ischemia modeling, NO production in the hippocampus decreases by an average of 30%, and there is a tendency to a decrease in NO in the frontal lobes and liver. The content of copper a day after modeling ischemia decreased in the frontal lobes by an average of three times and in the hippocampus by an average of 20%, while in the liver there was a tendency to decrease. Thus, cerebral hypoxia is accompanied not only by a decrease in NO production, but also by signs of a weakening of the antioxidant system in the hippocampus and frontal lobes, which additionally worsens the functional state of the homeostasis system.

Keywords: electron paramagnetic resonance, spin trap, nitric oxide, cerebral ischemia, frontal lobes, hippocampus

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INTRODUCTION

Nitric oxide (NO) is one of the key signaling molecules that regulate the physiological functions of the body, including the nervous system [1, 2]. NO is produced from the amino acid L-arginine by enzymes of the NO-synthase (NOS) family and is involved in the control of a number of cellular functions, including the regulation of blood vessel tone, neurotransmission, learning, immune response, and other functions [3]. In the life of animals and human beings, the role of NO in the functioning of the cardiovascular [4, 5] and nervous systems [1, 6] is especially significant. Great interest is posed by the participation of NO in the mechanisms of development of various pathological conditions of the body [4, 7, 8]. Accumulated evidence indicates that NO biosynthesis is one of the key factors in the pathophysiological response of the brain to hypoxia-ischemia [6, 9, 10]. NO performs its physiological functions by binding to iron (Fe) ions in the heme, or through S-nitrosylation of proteins, and also takes part in a number of biochemical reactions [11].

One of the reasons for the involvement of NO in the pathological process is a prolonged lack of oxygen, which leads to brain hypoxia. Hypoxia is accompanied by the development of tissue ischemia, which always occurs when the supply of body tissues with oxygen does not correspond to the real requirements of the tissues [6, 9]. With a decrease in the oxygen content in the inhaled air, disturbances in cerebral blood flow, leading to a lack of oxygen supply to the brain, cerebral ischemia occurs, which can result in an ischemic stroke, accompanied by damage to the brain tissues and its functions [12, 13]. In this regard, the study of the pathogenesis, methods of prevention and correction, and mechanisms of stroke is important from both the theoretical and practical point of views.

Currently, the development of cerebral ischemia and the subsequent occurrence of a stroke are associated with impaired cerebral blood flow, as well as with

violations of its regulation by the NO system [14, 15]. Unfortunately, there is still no consensus on the role of endogenous NO in the processes that occur during damage to the nervous system [16]. There is an explanation for this. There are a large number of methods for measuring NO production in biological systems. Recently, one of the most effective methods for detecting and quantifying of NO in biological tissues has become the method of electron paramagnetic resonance (EPR) [11, 17, 18]. This was due to a technique developed by Vanin et al., in which they used the spin-capture method based on the reaction of a radical (in this case, NO) with a spin trap [19]. As a result of the reaction, an adduct with a characteristic EPR spectrum is formed. It was found that the Fe^{2+} complex with diethyldithiocarbamate (DETC) upon NO capture forms a stable ternary complex $(DETC)_2–Fe^{2+}–NO$. These complexes are characterized by an easily recognizable EPR spectrum with *g*-factor $g = 2.035–2.040$ and a triplet hyperfine structure [11, 18]. The method has a sensitivity of 0.04–0.4 nM, allows direct measurements, and is highly sensitive due to the use of spin traps.

Thus, the dynamics of NO content in brain tissues during the onset and course of cerebral ischemia is still insufficiently studied, despite the recognition of the fact that the main contribution is made by NO produced by neuronal NOS (nNOS), and inducible NOS (iNOS) [1, 14, 20].

The aim of this work was to study the effects of experimental ischemic brain damage on the intensity of NO production in the frontal lobes, hippocampus, and liver of rats by EPR spectroscopy using the spin-trap technique.

1. EXPERIMENTAL TECHNIQUE

Modeling of ischemic brain damage was carried out in accordance with the approved protocol of the Commission on Ethics of the Institute of Physiology of the National Academy of Sciences of Belarus, Minsk. Experiments were carried out during daylight hours on 4-week-old male white rats (initial weight 139–145 g). The animals were kept under standard vivarium conditions (maintaining a 12/12-h rhythm of light and darkness, air temperature at $23 \pm 1°C$, and stable supply and exhaust ventilation) with free access to water and food (ad libitum) and a standard diet in accordance with the rules for keeping of laboratory animals.

Animals were divided into two groups: group 1 ($n = 10$), intact (control) rats, with the control group of animals not being subjected to surgical interventions and was tested under the same conditions as other groups of rats, and group 2 ($n = 10$), rats exposed to hypoxia (10-min blood-flow disturbance by ligation of both carotid arteries at the level of the vocal cords and taking 3 mL of blood from the common carotid artery). All surgical procedures, as well as tissue extraction, were performed on anesthetized animals (ketamine 55.6 mg/kg, xylazine 5.5 mg/kg, acepromazine 1.1 mg/kg intraperitoneally) [10, 21].

When preparing samples for measuring EPR spectra, we were guided by the spin-trap technique [17]. As before [22, 23], the spin-trap components for nitric oxide (DETC–Na, $FeSO_4$, sodium citrate) were introduced 30 min before the extraction of the studied tissues. The Fe^{2+} complex with diethyldithiocarbamate $(DETC)_2–Fe^{2+}$ was used as a spin trap. The spin-trap complex with NO ($(DETC)_2–Fe^{2+}–NO$) is characterized by an easily recognizable EPR spectrum with *g*-factor $g = 2.038$ and a triplet hyperfine structure [11, 17, 24]. DETC–Na was injected intraperitoneally at a dose of 500 mg/kg in 2.5 mL of water. A mixture of solutions—ferrous sulfate ($FeSO_4 \cdot 7H_2O$, Sigma, United States) was injected subcutaneously 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 weight), prepared immediately before injection, at three points—the right and left thigh and the rostral part of the interscapular region. The details of the experiment and technique were described earlier [23, 24]. Tissue samples were immediately frozen in liquid nitrogen and transported from Minsk to Kazan in a frozen state in plastic containers with dry ice. The complex of the spin trap with NO ($(DETC)_2–Fe^{2+}–NO$) is well preserved in this state, and the signal from the complex does not change for at least a month [18]. In addition, the spin trap interacts with Cu to form the $Cu(DETC)_2$ complex, which can also be detected by EPR spectroscopy [25].

The spectra of the $(DETC)_2–Fe^{2+}–NO$ and $Cu(DETC)_2$ complex were measured on Bruker X-range (9.50 GHz) EMX/plus spectrometers with an ER 4112HV and ER 200 SRC temperature attachment with a magnetic-field modulation of 100 kHz, modulation amplitude of 2×10^{-4} T, microwave power of 30 mW, time constant of 200 ms, and temperature of 77 K in a Bruker finger Dewar. The modulation amplitude, gain, and microwave power in all experiments were selected on the condition of the absence of overmodulation and saturation of the EPR signal, and these parameters remained the same throughout all measurements. Immediately before the measurement, the finished sample, truncated according to the shape of the measurement cuvette, was weighed. The weight of the samples was about 100 mg. The amplitude of the EPR spectra was always normalized to the weight of the sample and to the amplitude of the EPR signal of the reference sample (we described the details of the procedure for measuring EPR signals earlier [26]).

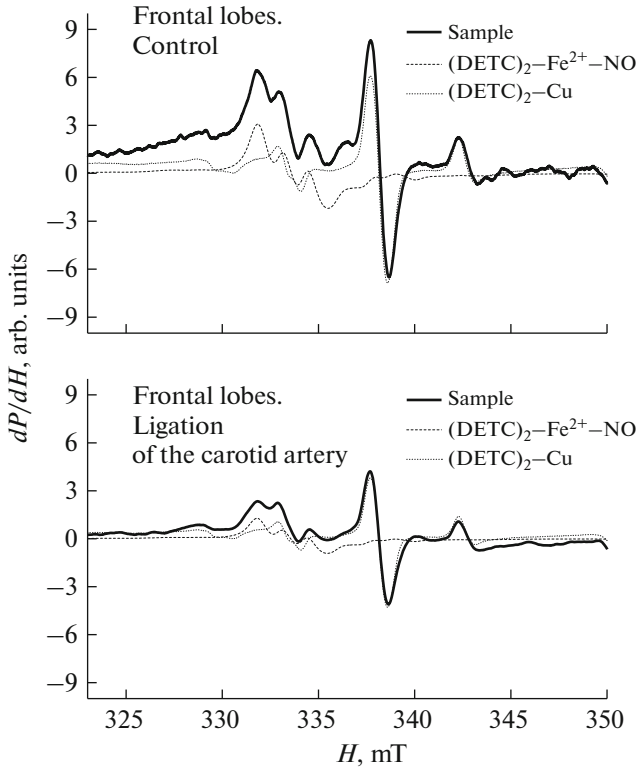

Fig. 1. Examples of EPR spectra of the frontal lobes of a control rat (above) and a rat after hypoxia caused by ligation of the carotid arteries followed by taking 3 mL of blood from the common carotid artery (below) a day after cerebral ischemia.The animals were injected with spin-trap components $(DETC)_2$–Fe^{2+}–citrate. $g_{av} = 2.038$.

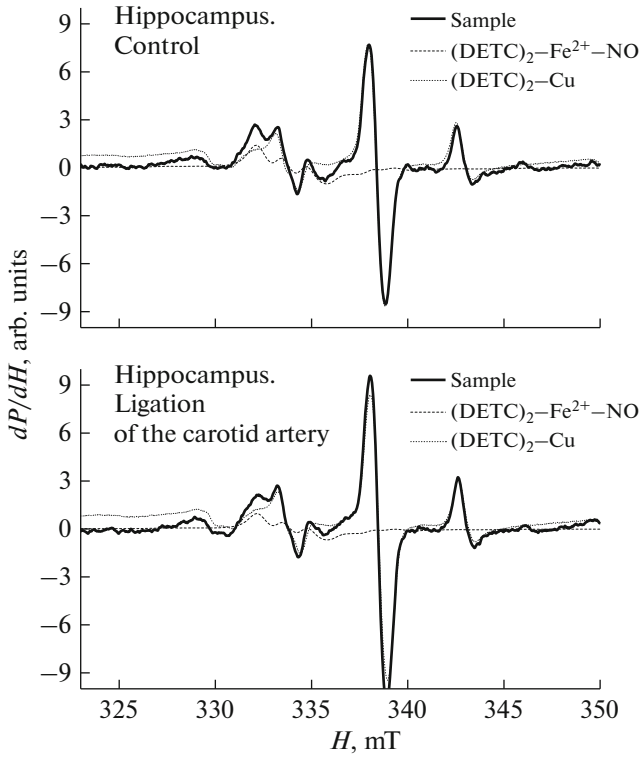

Fig. 2. Examples of EPR spectra of the hippocampus of a control rat (above) and a rat after hypoxia caused by ligation of the carotid arteries followed by taking 3 mL of blood from the common carotid artery (below) a day after cerebral ischemia. The animals were injected with spin-trap components $(DETC)_2$–Fe^{2+}–citrate. $g_{av} = 2.038$.

1.1. Statistical Processing of Results

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

2. RESULTS AND DISCUSSION

EPR spectroscopy was used to study the intensity of NO production and copper content (as an indicator of the first and third subunits of superoxide dismutase) in the frontal lobes, hippocampus, and liver during modeling of cerebral hypoxia caused by ligation of the carotid arteries for 10 min on both sides and taking a volume of 3 mL of blood from the common carotid artery.

Figure 1 shows the EPR spectrum of the frontal lobes of a control rat (top) and a rat after hypoxia caused by ligation of the carotid arteries, followed by taking 3 mL of blood from the common carotid artery (bottom) 1 day after cerebral ischemia. This spectrum shows a characteristic triplet signal from the $(DETC)_2$–Fe^{2+}–NO complex with a g-factor value of 2.038 [17]. In addition, in the same region, there is a signal from the $(DETC)_2$–Cu complex. Figure 2 shows the EPR spectra of hippocampal tissues of healthy rats (top), as well as rats 1 day after modeling (caused by ligation of the carotid arteries followed by taking 3 mL of blood from the common carotid artery) of ischemic stroke (bottom). The solid line represents the spectrum of the sample, the dashed line is the signal from nitric oxide bounded to the spin trap as part of the spectrum of the complex ($(DETC)_2$–Fe^{2+}–NO). The relative change in the amount of the NO-containing complex and the $Cu(DETC)_2$ complex was estimated from the integral intensity of the signal from these complexes.

Figures 3a–3c show statistical data on the integral intensities of the signal of $(DETC)_2$–Fe^{2+}–NO in the spectra of the studied samples of biological tissues, revealing the features of the spectra in ischemic stroke caused by ligation of the carotid arteries, followed by taking 3 mL of blood from the common carotid artery for evaluation NO production in brain tissues (frontal lobes and hippocampus) and liver 1 day after ischemia modeling. The results of the analysis demonstrate a decrease in NO production after modeling ischemic stroke in the hippocampus by an average of 30% ($p < 0.05$) and a trend towards a decrease in NO production

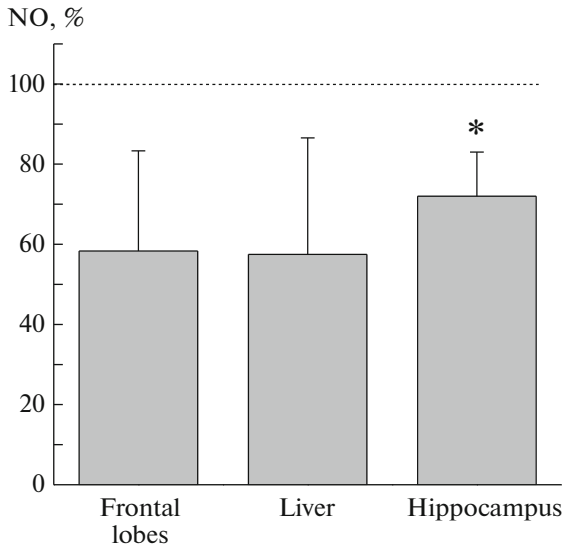

Fig. 3. NO content in the frontal lobes of the brain (frontal lobes), liver (liver), and hippocampus (hippocampus) when modeling hypoxia caused by ligation of the carotid arteries a day after ischemia. The ordinate axis—specific signal intensity of complexes $(DETC)_2$–Fe^{2+}–NO in animal-tissue samples after ischemia modeling as a percentage of complex signal intensity of $(DETC)_2$–Fe^{2+}–NO in the control group. *—difference from control (*t*-test, $p < 0.05$).

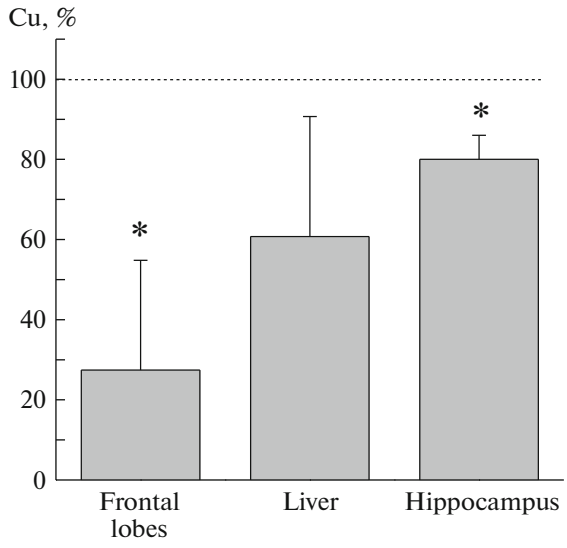

Fig. 4. Copper content in the frontal lobes of the brain (frontal lobes), liver (liver), and hippocampus (hippocampus) when modeling hypoxia caused by ligation of the carotid arteries a day after ischemia. The ordinate axis is the specific signal intensity of Cu complexes $(DETC)_2$ in animal tissue samples after ischemia modeling as a percentage of the signal intensity of Cu complexes $(DETC)_2$ in the control group. *—difference from control (*t*-test, $p < 0.05$).

in the frontal lobes and liver. The following Fig. 4 show the statistical data on the integrated signal intensities of $(DETC)_2$–Cu. The results show that the copper content 1 day after ischemia modeling significantly decreases in the frontal lobes by an average of three times ($p < 0.05$) and in the hippocampus by an average of 20% ($p < 0.05$), with a downward trend being noted in the liver. Thus, hypoxia is accompanied not only by a decrease in NO production, but also by a weakening of the antioxidant system in the hippocampus and in the frontal lobes, which further worsens the functional state of the nervous system.

Stroke is the leading cause of death and the most common cause of disability worldwide [12]. It is known that hypoxia is accompanied by disturbances in the supply of oxygen to brain regions; therefore, cerebral ischemia occurs, which often ends in ischemic stroke [27]. Previously, we performed experiments in which ischemic stroke was modeled in different ways: by 5-min hypobaric hypoxia, which was achieved by conditionally lifting the animals to a height of 4500 m above sea level [24], and by ligation of the common carotid arteries [8, 28]. In the work that was carried out, a more complex variant was used—ligation of the common carotid arteries was combined with taking 3 mL of blood from the common carotid artery. This study clearly demonstrated that the development of cerebral ischemia is accompanied by a decrease in the intensity of NO production.

On the one hand, the development of cerebral ischemia and the subsequent occurrence of a stroke are associated with a decrease in cerebral blood flow, as well as with disturbances in the regulation of blood supply to brain tissues by the NO system [6, 9, 14, 26, 29]. On the other hand, hypoxia itself resulting from ischemic stroke is accompanied by early cell death in various parts of the brain, followed by programmed late death of other brain cells by apoptosis [30]. In these processes of hypoxia–ischemia, the role of NO seems ambivalent: NO is able to perform both neurotoxic and neuroprotective functions [9, 16]. The reasons for the contradictory functions of NO are its synthesis by various NO synthases as the main source of NO [6, 14], the presence of the nitroreductase component of the NO cycle [4, 9], as well as a significant number of depots for NO that interact with complexes containing iron (for example, heme structures), with thiols and other compounds [10, 31, 32].

CONCLUSIONS

EPR spectroscopy was used to study the intensity of NO production and copper content (as an indicator of superoxide dismutase) in the frontal lobes, hippocampus, and liver of rats after modeling ischemic brain damage. These molecular components constantly attract the attention of researchers when studying the

mechanisms of brain functioning under normal and pathological conditions. It was shown that, 1 day after ischemia modeling, there is a decrease in NO production in the hippocampus and a tendency to a decrease in NO in the frontal lobes and liver, and the copper content significantly decreases in the frontal lobes and hippocampus and slightly ($p < 0.05$) in the liver. Thus, it can be assumed that hypoxia is accompanied not only by a decrease in NO production, but also by a weakening of the antioxidant system in the hippocampus and frontal lobes, which further worsens the functional state of the nervous system.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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