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Comparative Study of the Intensity of Nitric Oxide Production and Copper Content in Hippocampus of Rats After Modeling of Hemorrhagic Stroke and Brain Injury

V. V. Andrianov[1,2] · V. A. Kulchitsky[3] · G. G. Yafarova[1,2] · A. S. Zamaro[3] · Y. P. Tokalchik[3] · L. V. Bazan[1] · T. K. Bogodvid[2,4] · V. S. Iyudin[1] · S. G. Pashkevich[3] · M. O. Dosina[3] · K. L. Gainutdinov[1,2]

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Abstract

A comparative experimental analysis of intensity of nitric oxide (NO) production and the copper content in the tissues of hippocampus of male Wistar rats after modeling of hemorrhagic stroke and brain injury was conducted using EPR spectroscopy. Modeling of hemorrhagic stroke was carried out by microinjection of 500 nl of autologous blood into the brain to a depth of 5.0 mm (hippocampus) on the left side. Brain injury was performed by removing a piece of nerve tissue from 5.0 mm depth on the left side of hippocampus. It was registered a significant decrease in the NO content in hippocampus by $36 \pm 17\%$ on the 3rd day after modeling of hemorrhagic stroke together with decrease by an average of $24 \pm 14\%$ of the copper content. There were no significant changes in the NO level in hippocampus found neither on the 3rd day nor on the 7th day after brain injury modeling. There was also no change in copper content. Thus, it was experimentally demonstrated that modeling of brain injury, in contrast to hypoxia induced by hemorrhagic stroke, was not accompanied with significant changes in NO production in hippocampus of rat.

✉ K. L. Gainutdinov
kh_gainutdinov@mail.ru

[1] Laboratory of Spin Physics and Spin Chemistry, Zavoisky Physical-Technical Institute of the Russian Academy of Sciences, Sibirsky tract, 10/7, Kazan, Tatarstan 420029, Russian Federation

[2] Kazan Federal University, Kazan, Russian Federation

[3] Brain Center, Institute of Physiology, National Academy of Sciences, Minsk, Belarus

[4] Volga Region State University of Physical Culture, Sport and Tourism, Kazan, Russian Federation

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1 Introduction

The issue of restoring of neural brain networks after damage of different origin (trauma, strokes) has not yet been resolved [1–3]. According to the World Health Organization, annually more than 6 million people die from stroke across the world (the second leading cause of death) and more than 2 million people become disabled due to traumatic brain injuries. The pathological process that develops in brain after destruction of nervous tissue and impairment in control of vital somatic and visceral functions throughout the body has not yet been effectively tackled [1]. This is mainly due to insufficient studies about the mechanisms of pathogenesis of strokes and brain injuries [1–3].

Conditions for the proper functioning of neural networks are disrupted in brain injuries primarily due to mechanical damage to nervous tissue and blood vessels. In case of hemorrhages or ischemic strokes, blood supply to the brain is disturbed. Furthermore, in hemorrhagic strokes, a mechanical factor of nervous tissue compression in cranial cavity is added due to formation of hematomas. Ischemic stroke is characterized by brain tissue damage with hypoxia due to impaired blood flow in internal carotid arteries and/or vertebrobasilar region [4]. Thus, a number of similar processes are found in pathogenesis of brain injury and ischemic damage [5].

There is ample evidence that suggests impaired nitric oxide (NO) biosynthesis as the leading factor in pathophysiological response of brain to hypoxia–ischemia [4, 6, 7]. Cerebral ischemia is accompanied with accumulation of excitatory amino acids in brain tissues and activation of calcium-dependent isoforms of nitric oxide synthases (NOS) neuronal NOS (nNOS) and endothelial NOS (eNOS). However, while selective inhibition of nNOS is neuroprotective, selective inhibition of eNOS is neurotoxic.

Considering the above, the authors focused on relationship between the level of NO production in the body in stroke and brain injury. Both neuroprotective and neurotoxic effects of NO are noted, therefore, until now, there are conflicting opinions in scientific literature about the pattern of NO level correction in brain in pathology [7–9]. Endothelial dysfunction is often noted in brain injuries and strokes and it is manifested as a weakened endothelium-dependent relaxation of arteries, which is normally mediated by NO [10]. It is quite logical to compensate NO deficiency either by using NO donors or by activating NO synthesis in brain with pharmacological and non-pharmacological methods [6, 11–13].

The interest to NO role in brain functions is not accidental. NO is a gaseous chemical mediator that performs various functions in brain in many physiological processes including control of cerebral blood flow, features of interneuronal communication, synaptic plasticity, memory formation, receptor function, transmission of intracellular signals and release of neurotransmitters [14–18]. NO is involved in regulation of intracellular concentration of Ca^{2+} ions and pH regulation in cerebral ischemia by activating soluble heme-containing guanylate cyclase and ADP-ribosyltransferase [19, 20]. Depletion of NO, which is a basic mediator, leads to impaired microcirculation and blood flow regulation [10].

Several pathological mechanisms contribute to disruption of integrity of nerve and glial cells, as well as to destruction of extracellular matrix and damage to blood vessels in trauma and ischemia of the brain [10, 21, 22]. Similarities in pathogenesis of such cerebral injuries suggest that a therapeutic tactics protecting against cerebral ischemia in stroke may also be a viable choice for patients with brain injury. Cell damage mechanisms include glutamate excitotoxicity, oxidative stress, free radical production, apoptosis and inflammation [23–25]. Similarity of certain stages of pathogenesis of mentioned cerebral injuries indicates that therapeutic strategies of nervous tissue protection after ischemia may also be in demand after brain injury [11, 23–25]. All these processes radically change when nervous tissue is damaged as a result of trauma or stroke of ischemic or hemorrhagic nature. It is important to mention that neuroprotective effect of NO is closely related to its concentration, behavior of damaged brain cells and time after the onset of pathological reactions. Only when the appropriate conditions are fulfilled, the NO level correction can achieve protective effect [10, 21, 25, 26].

Activition of antioxidant enzymes are another method against highly toxic oxygen radicals. The most of them are associated with copper-containing enzymes [27, 28]. These are, first of all, Cu, Zn-SOD superoxide dismutase SOD1 and cytochrome c oxidase (CcO). CcO is an enzyme included in respiratory electron transport chain that catalyzes transfer of electrons from cytochrome c to oxygen. Dismutation of superoxide (O_2^-) by cytosolic enzyme SOD1 is the primary and main defense method against free radical oxidation processes [29]. It plays an essential role in antioxidant protection of almost all cells contacting with oxygen.

It is important from methodological point of view that one of the most effective methods for detection and quantitative determination of NO in biological tissues is a method of electron paramagnetic resonance (EPR) spectroscopy [19, 30]. In addition, it was shown that the spin trap interacts with Cu, forming the Cu^{2+}-$(DETC)_2$ complex, which can also be simultaneously detected by EPR spectroscopy [31]. Therefore, this EPR signal can serve as a relative indicator of the total level of copper-containing enzymes.

The study aims to compare intensity of NO production and copper content in hippocampus of rats under experimental conditions of hemorrhagic stroke and brain injury modeling.

2 Methods

2.1 Animals

The experiments were carried out on male rats ($n = 70$), weighing 200–250 g. The animals were kept under standard vivarium conditions with ad libitum access to food and water. Modeling of hemorrhagic stroke and brain trauma were performed at the Brain Center of the Institute of Physiology of the National Academy of Sciences of Belarus, Minsk. The modeling was carried out in accordance with the approved protocol of the Ethics Commission (protocol No. 1 dated January 31, 2019) of the Institute of Physiology of the National Academy of Sciences of Belarus, Minsk. Brain

tissue samples in both models were taken in three and seven days after injury and stroke ($n=10$ in each series); there was also control group of intact rats ($n=10$). These times were chosen for two reasons: on the one hand, these are previously conducted experiments with immunohistochemical staining of damaged brain areas [32], and on the other hand, these are behavioral experiments in which we showed that intranasal administration of mesenchymal stem cells leads to rapid recovery of motor activity in experimental animals [33]. Biological material in special containers was sent from Minsk to Kazan. 20 animals remained in Minsk (10 after brain injury modeling and 10 after stroke modeling) for 1-month follow-up to assess effectiveness of restoration of central control of motor functions.

2.2 Experiment Protocol. Brain Injury Modeling in Rats

All surgical procedures were performed on anaesthetized animals (55.6 mg/kg ketamine, 5.5 mg/kg xylazine, 1.1 mg/kg acepromazine, injected intraperitoneally) [34]. Modeling of brain injury was carried out by removing a section of nerve tissue (volume 2.5 mm^3) using aspiration through a burr hole ($d=1.2$ mm), which was bored according to the following stereotaxic coordinates [35]: 6.0 mm caudal to the bregma, 5.0 mm lateral to the midline, at a depth of 5.0 mm (hippocampus) on the left side. Tissue samples were taken in three ($n=10$) and seven ($n=10$) days after brain injury modeling.

2.3 Hemorrhagic Stroke Modeling in Rats

Modeling of hemorrhagic stroke was carried out by microinjection of 500 nl of autologous blood (taken from the tail vein of laboratory rat) through a burr hole ($d=1.2$ mm) according to the stereotaxic coordinates [35]: 6.0 mm caudal to the bregma, 5.0 mm lateral the middle line, to a depth of 5.0 mm (hippocampus) on the left side. Tissue samples were taken in three ($n=10$) and seven ($n=10$) days after hemorrhagic stroke modeling.

The control group of animals ($n=10$) did not undergo surgery and was tested under the same conditions as other groups.

2.4 Formation of the Ternary Complex $(DETC)_2$-Fe^{2+}-NO in Rat Tissues

The main difficulty in determining the content of NO in free state in tissues and body fluids is its extreme activity and short lifetime resulting low concentration. There are several methods for measuring NO production in biological systems [36], including method of electron paramagnetic resonance (EPR) [30, 37]. EPR spectroscopy has become one of the most effective methods for detection and quantification of NO in biological tissues [19, 30, 36]. The method is based on formation of Fe^{2+} complex with diethyldithiocarbamate (DETC) to capture NO and form a stable ternary complex $(DETC)_2$-Fe^{2+}.

Previously tested spin trap technique [38] was used in this study: DETC-Na was injected intraperitoneally at the dose of 500 mg/kg in 2.5 ml of water [39, 40]. A

mixture of solutions: ferrous sulphate ($FeSO_4 \times 7\ H_2O$, Sigma, USA) at the dose of 37.5 mg/kg and sodium citrate at the dose of 187.5 mg/kg (in 1 ml of water per 300 g of animal weight), was prepared immediately before injection and injected subcutaneously at three sites—right and left thighs and in rostral part of interscapular region. Ferrous citrate was formed in a mixture of ferrous sulphate and sodium citrate. DETC-Na and iron citrate were distributed throughout the body and interact to form a water-insoluble DETC-Fe^{2+} complex. The spin trap complex with NO is characterized by easily recognizable EPR spectrum with a *g*-factor $g = 2.038$ and a triplet hyperfine structure [19]. In addition, the spin trap interacts with Cu, forming the complex Cu^{2+}-$(DETC)_2$, which can also be detected by EPR spectroscopy [31].

Areas of brain damage in CA1 area of hippocampus and similar brain area from rats of the control group weighing 100 mg were taken after decapitation and trepanation within several seconds, packed into insulin syringe and placed in container with liquid nitrogen. The inner diameter of syringe coincides with the inner diameter of the finger Dewar where measurements were carried out, so the tissue samples were easily used for measurements.

2.5 Measurement of EPR spectra of $(DETC)_2$-Fe^{2+}-NO and Cu^{2+}-$(DETC)_2$ complexes in rat tissues

The spectra of $(DETC)_2$-Fe^{2+}-NO and Cu^{2+}-$(DETC)_2$ complex were measured using an EMX/plus X-band (9.50 GHz) spectrometer with temperature controller Bruker ER 4112HV at the temperature of 77° K with magnetic field modulation 100 kHz, modulation amplitude 2Gs, microwave power 30 mW, time constant 200 ms in a Bruker cold finger quartz Dewar. Modulation amplitude, amplification, and microwave power were selected to exclude overmodulation and saturation of EPR signal in all experiments and remained the same throughout all measurements. Samples' weights were about 100 mg. The amplitude of EPR spectra was normalized to sample weight and amplitude of EPR signal of reference sample with known concentration (details of procedure for EPR signals measurement were described earlier [9, 41].

The intensity of absorption by complexes $(DETC)_2$-Fe^{2+}-NO and Cu^{2+}-$(DETC)_2$ was evaluated by selecting the optimal amplitudes of their reference signals (with obtaining the smoothest remainder of the curve in this region). This method is close to peak-to-peak analysis, but it has an advantage in the case of overlapping signals [31, 42]. The reference signal of the complex $(DETC)_2$-Fe^{2+}-NO was chosen by us when analyzing experimental biological samples in which no other signals were observed except for the triplet $(DETC)_2$-Fe^{2+}-NO, and there was no overlap of other signals in this area. In addition, this reference signal of the complex $(DETC)_2$-Fe^{2+}-NO was compared with the signal from the sample obtained by chemical means, by the method of interaction of the applied spin trap with NO isolated from sodium nitroprusside, as well as with the described signal form $(DETC)_2$-Fe^{2+}-NO in the literature [19, 39]. We did not find any differences in the form of these signals. The EPR spectrum of the complex Cu^{2+}-$(DETC)_2$ consists of four lines ($g\perp = 2.025$) corresponding to a magnetic hyperfine structure—quarter [42, 43]. When analyzing

the intensities, changes and differences in the shapes of the reference and experimental signals were neglected. Indeed, we did not find any obvious changes in the shape of these signals. A sign of the optimal selection of the amplitude of the reference signals was the smoothness of the curve remaining after subtraction, which was controlled by the least squares method. The selection of the amplitudes of the standards began with the signal of Cu^{2+}-$(DETC)_2$ using its extreme high-field hyperfine component with $g = 1.983$, there is no signal overlap in this region. Then from the remaining curve after subtracting the signal from Cu^{2+}-$(DETC)_2$, the amplitude of the reference signal was selected from $(DETC)_2$-Fe^{2+}-NO, optimal for obtaining the smoothest remainder (ideally, the zero line) in the region $g = 2.055–2.020$.

2.6 Statistical Processing

The results are presented as M ± SEM (mean ± standard error of the mean). Statistical data processing was performed using Student's t-test in an experiments with modeling of hemorrhagic stroke (two groups of animals). One Way ANOVA statistics were applied in an experiment with modeling brain injury (three groups of animals). Differences were considered significant at $p < 0.05$.

3 Results

Modeling of local brain injury (by unilateral aspiration of hippocampus fragment at the level of CA1 region) or hemorrhagic stroke (by injecting 500 nl of autologous blood at the level of CA1 region of hippocampus) in animals resulted in impairment of central control of motor activity. The following was detected in 1 day after modeling of brain injury or stroke: rats repeatedly incautiously approached the edge of elevated cruciform labyrinth and sometimes fell down from the height of the maze on the floor, showed impaired movements in open and closed zones of labyrinth; all these were frequently recorded earlier in our experiments [44]. Patients with brain trauma or strokes of hemorrhagic or ischemic nature also exhibit gross violations of central control of motor, visceral and cognitive functions in clinical practice [45]. The question arises: how dynamically the NO system reacts in functional or pathophysiological aspects in nervous tissue damage? It is also important to clarify the features of NO system reactivity in brain in traumatic injury and development of hemorrhagic stroke.

Figure 1 showed typical EPR spectra recorded from the tissue of hippocampus in control rat and rat on the 3rd day after modeling of hemorrhagic stroke. EPR signal from NO was represented by a triplet in the region of 330–337 mT with a g-factor of 2.038. Also, there was a well-expressed signal from the copper complex with DETC - Cu^{2+}-$(DETC)_2$. Figure 2 showed the ratio of amplitudes of $(DETC)_2$-Fe^{2+}-NO in the spectra of hippocampal samples of control animals (taken for 100%) and animals after hemorrhagic stroke. The averaged data of the experiments of hemorrhagic hypoxia modeling by introducing the animal's autologous blood with micropipette in CA1 region of hippocampus on one side

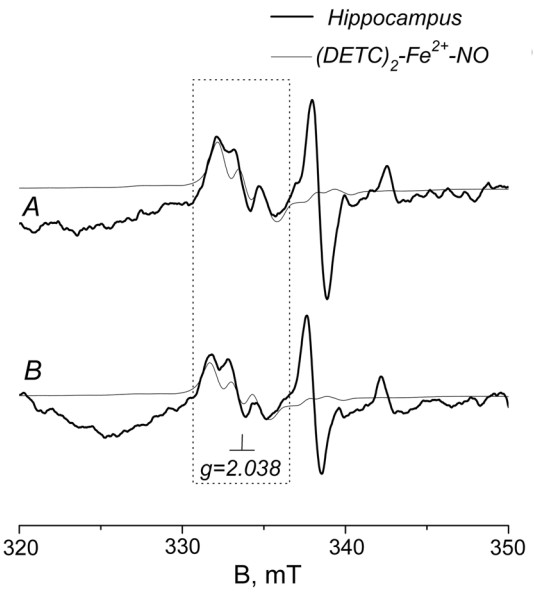

Fig. 1 EPR spectra of the hippocampal tissues in the control rats (upper figure) and the rats on 3rd day after modeling hemorrhagic stroke (lower figure). Signals from: **Thick line** tissue sample (hippocampus), **Thin line** calculated spectrum of the $(DETC)_2$-Fe^{2+}-NO complex from observed spectrum. The dotted line shows the contribution of NO to the observed signal. Temperature is 77° K. The rats were injected with $(DETC)_2$-Fe^{2+}-citrate. $g_{cp} = 2.038$

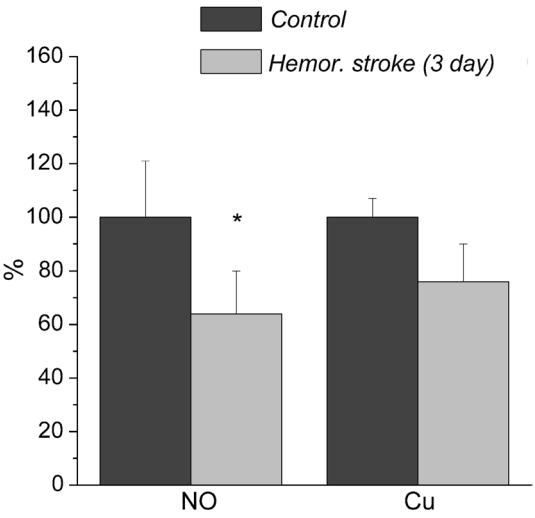

Fig. 2 The relative content of NO and copper in the hippocampus of healthy rats (Control) and rats on 3rd day after modeling hemorrhagic stroke. It is shown the mean intensity of the integrated signal $(DETC)_2$-Fe^{2+}-NO and Cu^{2+}-$(DETC)_2$ in relative to control (100%). (*) indicates a significant difference of NO content between Control and Insult groups ($p < 0.05$)

(cerebral hemorrhage modeling) showed significant decrease in NO production in hippocampus on 3rd day after modeling of hemorrhagic stroke by $36 \pm 17\%$ ($p = 0.045$) and decrease in copper content by an average of $24 \pm 14\%$ ($p = 0.082$) (Fig. 2). Figure 3 demonstrated an example of EPR spectrum of hippocampus of intact (control) rats and rats in 3 days after brain injury. EPR signal from NO was represented by triplet in the region of 330–337 mT with a g-factor of 2.038. Also, there was a well-expressed signal from the copper complex with DETC Cu^{2+}-$(DETC)_2$. Figure 4 demonstrated that there was no significant changes in

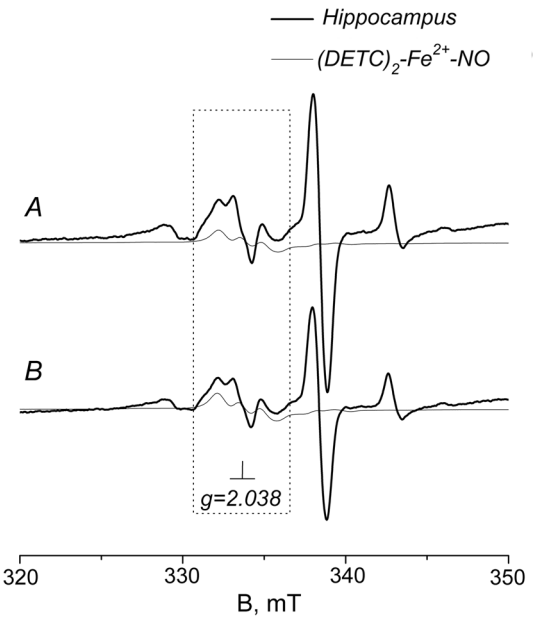

Fig. 3 EPR spectra of the hippocampal tissues in the control rats (upper figure) and the rats 3 days after modeling brain injury (lower figure). Signals from: **Thick line** tissue sample (hippocampus), **Thin line** calculated spectrum of the $(DETC)_2$-Fe^{2+}-NO complex from observed spectrum. The dotted line shows the contribution of NO to the observed signal. Temperature is 77° K. The rats were injected with $(DETC)_2$-Fe^{2+}-citrate. $g_{cp} = 2.038$

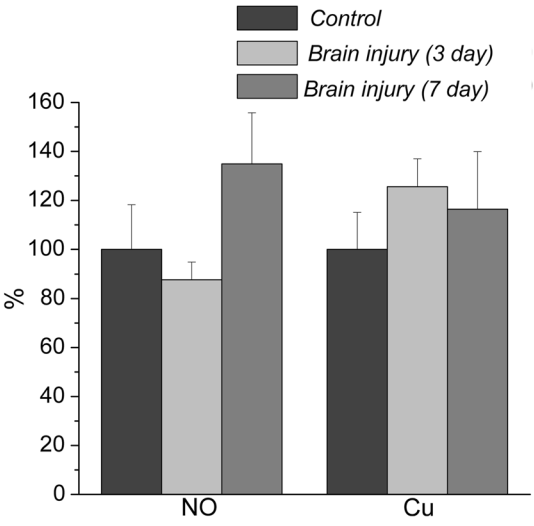

Fig. 4 The relative content of NO and copper in the tissues of the hippocampus of healthy rats (Control) and rats on 3rd and 7th days after modeling brain injury. The ordinate axis represents the mean intensity of the integrated signal $(DETC)_2$-Fe^{2+}-NO and Cu^{2+}-$(DETC)_2$

NO level in 3 and 7 days after modeling of brain injury in hippocampus. Statistical analysis of ANOVA did not reveal a difference between groups in the case of the signal $(DETC)_2$-Fe^{2+}-NO ($p = 0.172$), and in the case of signal of the Cu^{2+}-$(DETC)_2$ ($p = 0.644$). The spectroscopic characteristics of the complexes in our experiments are closes to those obtained in [31, 43]. $g\perp = 2.025$ for Cu^{2+}-$(DETC)_2$ and $g = 2.035$ for $(DETC)_2$-Fe^{2+}-NO. The EPR signal form

for this complex is a triplet hyperfine structure. These data were obtained earlier [39].

4 Discussion

Prolonged shortage of oxygen leads to brain hypoxia [4, 46], which, under certain conditions, is accompanied with development of tissue ischemia when oxygen supply to body tissues does not match what the tissues actually need due to natural process of biological oxidation. Ischemia is an important component of pathogenesis of many diseases [23, 47]. Such disturbances can occur in post-traumatic stress disorder characterized by repeated reminiscences of memory of original traumatic event. Disorders of cerebral blood flow, which lead to lack of oxygen supply to brain regions, also lead to cerebral ischemia, which can result in ischemic stroke accompanied with damage of brain tissue and its functions [23, 48]. Impaired oxygen supply of the brain also occurs when a vessel is thrombosed or an aneurysm ruptures leading to ischemic or hemorrhagic stroke [11, 49]. NO plays both protective and destructive role in these pathological processes, being controlled by many factors that ultimately determine involvement of various forms of NO synthases in the process [8, 11].

EPR spectroscopy allowed us to study intensity of NO production and copper content in hippocampus of rats after modeling of hemorrhagic stroke and brain injury. These molecular components have been attracting attention of researchers for a long time when studying the mechanisms of brain functioning and pathological disorders. Various methodological approaches are used to study them [34], and one of the most sensitive is the method of EPR spectroscopy [19, 30, 50]. Significant progress in this method was achieved after development of the electron spin trap method by prof. A. F. Vanin and his colleagues for detection and quantitative determination of NO in biological tissues [19]. The spin capture method is based on reaction of radical (NO in this case) with a spin trap. An adduct with characteristic EPR spectrum is formed as a result of this reaction. It was also shown that the spin trap interacts with Cu, forming the Cu^{2+}-$(DETC)_2$ complex, which is also determined by EPR spectroscopy [31, 42]. The reason for formation of these complexes is high affinity of Cu^{2+} ions to oxygen or imidazole groups of aspartic/glutamic acid or histidine [51]. Affinity of its binding to DETC was estimated to be significantly higher than other bonds [27, 42]. The absence of significant changes in copper content in our experiments with both modeling of hemorrhagic stroke and brain injury demonstrates stability of copper homeostasis in brain in these models [27, 42]. Importantly, DETC used as a spin trap has a potent cooper-chelating capacity, yielding Cu(II)-DETC complexes that are more stable then other DETC complexes with divalent metal ions, for example Fe(II) [42, 43, 52]. This strong affinity of DETC for Cu opens up a potential therapeutic avenue.

The results obtained in these experiments demonstrated that hemorrhagic cerebral stroke was characterized by reduced NO production in hippocampus. We would like to mention that we had previously shown a decrease in NO production

in the model of ischemic stroke when cerebral hypoxia was caused by ligation of carotid arteries [53]. In particular, we had demonstrated in our experiments that brain injury modeling, in contrast to hypoxia in hemorrhagic stroke, was not accompanied with significant changes in NO in hippocampus of rat's brain. This result differed from the data presented in the review by Garry et al., 2015, which shows a decrease in NO after traumatic brain injury. A rapid drop in NO concentration in damaged brain below the control values was also found, was persisted for several hours and varied depending on the brain region [50]. Interestingly, this decrease in NO concentration was accompanied with simultaneous increase in the number of NADPH-diaphorase-positive cells, which may indicate an increase in NOS activity. This result may explain contradiction in results of measuring NO content and NOS activity. It is assumed that very rapid removal of NO occurs in damaged brain by its transformation into other active forms [50].

Primary and secondary brain injuries caused by stroke have complex pathophysiological processes including inflammatory response, neuronal apoptosis, ischemia–reperfusion injury, free radical generation, etc. [24, 48, 54]. On the one hand, development of cerebral ischemia and subsequent onset of stroke are associated with impaired regulation of blood supply to brain tissues and dysregulation of NO system [4, 7, 9, 10, 26, 47]. On the other hand, hypoxia resulting from stroke is directly accompanied with early cell death in various parts of the brain, followed by programmed late death of other cells by apoptosis [24, 55].

Not only neurons and glial cells are damaged in brain injury, but also blood vessels [10]. The change in blood supply to the damaged brain area is accompanied with enhancement of destructive processes, which actually become dominant in post-traumatic period when direct effect of physical factor that caused the injury is no longer present. Spontaneous restoration of damaged vessels and the onset of reperfusion require a certain amount of time. However, our results showed that molecular mechanisms of these two types of pathologies did not match. This was especially true for the NO system. Comparative analysis of EPR spectra of hippocampus after modeling of brain trauma and stroke of hemorrhagic nature demonstrated the presence of both distinctive features in the spectra and virtually identical changes in their individual sections (Figs. 1, 3). Measurements of NO content in rat's hippocampus after modeling of brain injury showed no changes both on the 3rd and 7th days after the injury.

Considering the low effectiveness of existing therapy tactics to treat brain injuries and strokes, despite numerous debates about reperfusion period, it is sensible to study in more depth the mechanisms and therapeutic possibilities of existing therapy methods. It is also advisable to take into account the data of our experiments that suggest significant differences in dynamics of nitrosyl stress and the state of antioxidant defence when tackling brain trauma and hemorrhagic stroke as they form an experimental basis for development of new tactics in this area of medicine.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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