14

EPR spectroscopy study of NO and copper content in rat liver after combined brain and spinal cord injury

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Electron paramagnetic resonance (EPR) spectroscopy was used to determine the nitric oxide (NO) and copper content in rat liver tissue after combined brain and spinal cord injury. The spin trap method was used to record signals from $(DETC)_2$-Fe^{2+}-NO and $Cu(DETC)_2$ complexes. Direct EPR spectroscopy measurements revealed that the NO production in the liver decreased reliably by a factor of 2 seven days after modeling of combined brain and spinal cord injury. The copper content in the liver did also decrease, but these data were statically unreliable.

Keywords: electron paramagnetic resonance, spin trap, nitric oxide, combined brain and spinal cord injury, liver.

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Introduction

Nitric oxide (NO) is a highly reactive free radical that acts as both an oxidizer and a reducer in various biochemical processes [1,2]. The discovery of the ability of mammalian cells to synthesize NO has stimulated enormous research efforts aimed at investigating the role of NO in various fields of biology and medicine [2]. Being a key signaling molecule, NO is involved in the regulation of numerous physiological functions of the body, including the nervous system [3]. NO is ubiquitous in the nervous [4,5], cardiovascular [6,7], and other functional systems of the body and has a role in the regulation of metabolism, vascular tone, neurotransmission, learning, and a number of other functions [3,8–10]. In addition to its vasodilatory, neurotransmitter, and stress-limiting properties, NO has been demonstrated to be involved in oxidative stress reactions, the calcium glutamate cascade, and inflammatory processes [4,11,12].

Of particular interest is the involvement of NO in the development of various pathological conditions of the body [13,14,15]. There is considerable evidence that impaired NO biosynthesis is the driving factor in pathophysiological response of the brain to hypoxia and ischemia [16,17,18]. Long-term oxygen deficiency leading to brain hypoxia is one of the reasons why NO is involved in the pathological process [16,19]. It is fundamentally important to bear in mind that coordinated functioning of the NO system is disrupted in the case of cerebral hypoxia and ischemia [20,21,22]. Oxygen supply to various parts of the brain is also disrupted when a blood vessel becomes thrombosed or an aneurysm ruptures, which often results in an ischemic or hemorrhagic stroke [16]. The ambivalent nature of NO is revealed again in the processes proceeding in the brain during hypoxia and ischemia: the current belief is that it may perform both neurotoxic and neuroprotective functions [18,22,23]. There is growing evidence that NO plays an important neuroprotective role in strokes, even though NO is usually regarded as a toxic gas. Therefore, we need to take a dialectical approach to NO, and further research (animal and clinical studies included) may provide new insights into the treatment of stroke and other central nervous system diseases [11,21].

It is commonly accepted at present that oxidative stress is involved in the molecular and cellular mechanisms of pathological conditions of the brain [24]. Oxidative stress (OS) induced by unfavorable exogenous factors or the activation of endogenous mechanisms for generation of reactive oxygen and nitrogen intermediates and weakening of the antioxidant defense of the body is currently regarded as an important pathogenetic link in development of numerous diseases [25,26]. OS is the result of a sharp intensification of oxidative processes in the body with insufficient functioning of the antioxidant system [27,28]. The human antioxidant defense is a complex system that establishes a physiologically important level of reactive

oxygen intermediates (ROIs) in the cell to maintain cellular signaling, while also minimizing ROI levels to prevent oxidative damage [12]. Much attention is paid to the assessment of the OS level, since the involvement of OS mechanisms in pathogenesis of numerous diseases may be attributed to the universality and critical importance of oxidation-reduction reactions in cells of the body both under normal conditions and in the course of typical general pathological processes [24–26,28]. Alongside with ROIs, reactive nitrogen intermediates (primarily NO) and metals of variable valence (Cu and Fe) contribute actively to the development of OS [12,24,29–31].

Traumatic brain injury (TBI) is a major healthcare and social issue in all countries throughout the world. In terms of the number of years of life lost, it leaves its „competitors" far behind, exceeding the mortality rate from cardiovascular diseases (cancer) at a young age by a factor of 10 (20). In almost 60% of cases, the cause of death is damage to the brain itself [32,33]. „Among all causes of death, TBI occupies one of the leading positions. The average incidence rate of TBI is 3−4 cases per 1 000 people. In Russia, about 600 000 people suffer from TBI every year; 50 000 of them die, and another 50 000 become officially disabled" [34]. The mechanism of injury in TBI needs to be understood in order to develop prophylactic procedures and establish rules (e.g., in sports) aimed at preventing the incidence of such injuries. Human variability and the inability to examine these injury mechanisms directly have led to the study of TBI in animal models and via physical and numerical modeling in an attempt to understand the underlying injury mechanics [35].

TBI cases are common worldwide and are associated with high levels of disablement and incapacitation. It is generally recognized that the highest TBI incidence rates are observed at a very young age (0−4 years) and in adolescents and young adults (15−24 years) [36,37]. Brain injury is damage to the brain induced by an external mechanical force, which may lead to temporary or permanent impairment [38,39]. TBI is characterized by spasms of blood vessels and diffuse or focal damage to brain tissue [36,40]. It was demonstrated that the primary injury is followed by an evolving cascade of secondary injuries [35,37,40,41]. There is evidence that NO plays an important and multifaceted role in these processes. Studies suggest that the functional role of endogenous NO in the processes associated with damage to the nervous system is ambiguous and insufficiently studied [16,18,31,41–44]. In other words, NO is akin to two-faced Janus from ancient Rome [23,45]. Such studies remain relevant, since they help solve socially important issues of increasing the average life expectancy of the population with hemorrhagic and ischemic strokes and combined brain and spinal cord injury.

Thus, we conducted an EPR spectroscopy examination of the liver of rats with combined brain and spinal cord injury and assessed the NO production (as an indicator of the overall level of NO production in the body) and the copper content (as an indicator of the first and third subunits of superoxide dismutase) in it.

1. Experimental procedure

1.1. Inducing combined brain and spinal cord injury in rats

Combined brain and spinal cord injury was induced at the Brain Center of the Institute of Physiology of the National Academy of Sciences of Belarus in Minsk in accordance with protocol No. 1 approved by the Ethics Commission of the Institute of Physiology on January 31, 2019 (approval code E7/04/2023). Experiments were performed in daylight hours on male rats ($n = 20$) weighing 200−400 g. Animals were kept under standard vivarium conditions (with a 12/12-hour rhythm of light and darkness, an air temperature at the level of $23 \pm 1\,^{\circ}\mathrm{C}$, and stable supply and exhaust ventilation) with free access to water and food (*ad libitum*) and a standard diet in accordance with the standards for management of laboratory animals.

Brain injury was induced first in the precentral region of the brain on the left (frontal lobe), and then spinal cord injury was induced at the level of the first lumbar vertebra. Anesthetized laboratory rats were fixed by limbs on a surgical table in a pronated position. The periosteum was removed locally in the projection of the precentral gyrus, craniotomy was performed with a dental drill, and local brain damage was induced with a stylet in the precentral region on the left (this took 3−4 min). At the next stage, the operation was continued at the level of the lumbar spinal cord. The stylet was inserted into the spinal cord at the level of the first lumbar vertebra, and the duration of bleeding from the wound after removal of the stylet was noted in the diary. The procedure was detailed in [46]. If we go by the classical classification of wounds [34,39], this is not a compression, not a contusion, and not a concussion; in essence, this is a penetrating wound to tissues of the brain and the spinal cord. The control group of animals did not undergo surgery. All surgical procedures were performed on anesthetized animals (55.6 mg/kg of ketamine, 5.5 mg/kg of xylazine, 1.1 mg/kg of acepromazine intraperitoneally) [47]. Liver samples were retrieved seven days after surgery $n = 5$), and the same number of animals ($n = 5$) were left undisturbed to assess the efficiency of restoration of central control of motor functions after surgery. Tissue samples were also taken from intact rats ($n = 5$, control group), and five animals were left for evaluation of motor functions. These time intervals were chosen for two reasons: on the one hand, it was demonstrated in earlier experiments with immunohistochemical staining on modeling of a local area of neurodestruction in the sensorimotor zone of the brain and the introduction of mesenchymal stem cells (MSCs) into the submucosal region of the nasal cavity of rats that stem cells move along the *n. olfactorius* fibers into the central structures of the olfactory analyzer and are distributed in damaged brain areas in the anterior cranial fossa [48,49]. On the other hand, these are behavioral experiments: it was found that perineural administration of MSCs to rats in the acute period of cerebral ischemia

was accompanied by objective signs of recovery of cognitive and motor functions one and three days after surgery; on the seventh day after modeling of ischemia in rats with the administration of MSCs, there were virtually no differences in control of motor activity compared to the same rats in the preoperative period [50,51]. The biological material was transported from Minsk to Kazan in a dewar with liquid nitrogen, where it was stored until measurements could be made. Ten animals subjected to surgical modeling of brain and spinal cord injury were left in Minsk and observed for a month after the start of the experiment to assess the efficiency of restoration of central control of motor functions.

1.2. Determination of nitrogen oxide and copper by EPR spectroscopy

A number of methods for measurement of the NO production in biological systems are known [52]. EPR has recently become one of the most efficient methods for determination of NO in biological tissues [53–56], which is the result of studies performed by A.F. Vanin et al. [57] where the so-called spin trapping method was refined. In 1984, Vanin et al. have proposed the use of a complex of divalent iron with diethyldithiocarbamate (DETC) as a trap for NO in animal cells and tissues. The method relies on the formation of a complex of Fe^{2+} with DETC and trapping of NO in a stable ternary $(DETC)_2$-Fe^{2+}-NO complex. In other words, it is based on the reaction of a radical (in the present case, NO) with a spin trap. With this spin trap, the method measures the overall NO level in the examined tissue sample (i.e., the sum of both free NO and its stabilized forms, such as *S*-nitrosothiols, dinitrosyl iron complexes, etc.) [45,58]. The authors of the original technique suggested the use of ultrahigh concentrations of NO traps in cells and tissues for NO determination [53,58]. This approach allows one to measure the maximum amount of NO, but entails a major disturbance of cellular metabolism [59,60].

To form a spin trap, 500 mg/kg of DETC-Na were administered intraperitoneally (in 2 ml of water per 300 g of animal weight). A mixture of solutions of iron sulfate ($FeSO_4 \cdot 7H_2O$, Sigma, United States) in a dose of 37.5 mg/kg and sodium citrate in a dose of 187.5 mg/kg (in 1 ml of water per 300 g of animal weight), which was prepared immediately prior to administration, was then injected subcutaneously at three points: the right and left thighs and the rostral part of the interscapular region [14,61]. All components were administered 40 min before the rats were sacrificed and the examined organs were isolated. Iron citrate is produced in a mixture of iron sulfate and sodium citrate. DETC-Na and iron citrate are distributed throughout the body with blood flow, forming a water-insoluble compound. If NO is available, a paramagnetic $(DETC)_2$-Fe^{2+}-NO complex, which is stable and persists for quite a long time, forms next. The half-life of this molecule at room temperature is close to 1.5 h [58]. The $(DETC)_2$-Fe^{2+}-NO complex of the spin trap and NO is characterized by an easily recognizable EPR spectrum with a *g*-factor of $g = 2.038$ and three hyperfine structure components [58,62,63]. In addition, the spin trap interacts with Cu and forms a $Cu(DETC)_2$ complex that may also be detected by EPR spectroscopy [63,64].

An X-band (9.5320 GHz) EMX/plus spectrometer from Bruker was used to measure the spectra of $(DETC)_2$-Fe^{2+}-NO and Cu^{2+}-$(DETC)_2$ complexes in biological samples. The examined sample in a Bruker finger-type dewar was introduced into the cavity of a double resonator (model ER 4105DR), and the reference sample was positioned in the other cavity of the same resonator. Since the experimental conditions were identical, the signal intensities of the two samples could be compared (with the change in g-factor during measurements taken into account). The magnetic field modulation frequency was 100 kHz, the modulation amplitude was 2 G, the microwave radiation power was 2 mW, the time constant was 327 ms, and the measurement temperature was 77 K. The modulation amplitude and the microwave amplification and power were set in all experiments in such a way as to avoid overmodulation and saturation of the EPR signal, and these parameters were kept unchanged throughout the measurements. The weight of samples was 150−200 mg. The EPR spectra amplitude was normalized to the sample weight.

1.3. Statistical processing of the result

The result is presented as $M \pm m$ (mean value — standard error of mean). Statistical processing of the obtained data was performed using the Student's *t*-test with verification of normality and equality of variances. Differences were considered to be significant at $p < 0.05$.

2. Results

EPR spectroscopy was used to examine the intensity of NO production and the copper content (as an indicator of the first and third subunits of superoxide dismutase) in rat liver after combined brain and spinal cord injury. The body of animals contains a significant number of copper-containing enzymes [65,66]. One of them is superoxide dismutase Cu, Zn-SOD (SOD1) [66,67,68]. The first and third subunits of superoxide dismutase contain copper as a transition metal: Cu, Zn-SOD (copper as a cofactor of the active center and zinc as a cofactor stabilizing the conformation) [69]. The neutralization of superoxide free radicals (O_2^-) by the SOD1 cytosolic enzyme is the primary protection against free radical oxidation processes [70].

Figure 1 presents the example EPR spectra of liver tissue from a control rat (top) and liver tissue sampled seven days after combined brain and spinal cord injury. These spectra were measured with microwave radiation of constant frequency under magnetic field sweep. Signals from different paramagnetic particles (complexes) are visible. A signal from the $(DETC)_2$-Fe^{2+}-NO spin trap complex

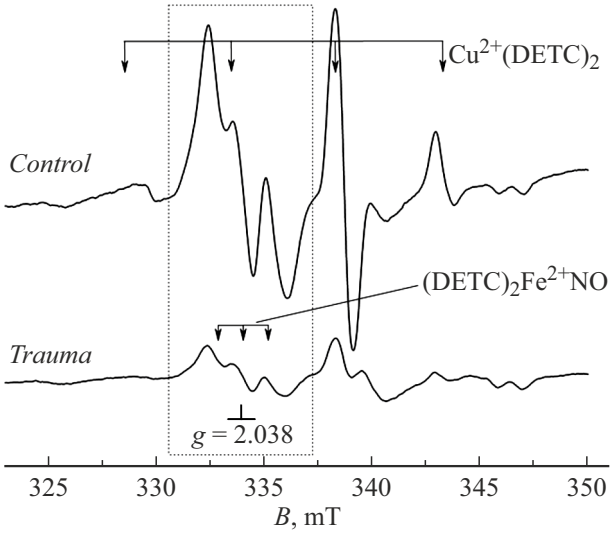

Figure 1. Example EPR spectra of liver tissue from a control rat (top) and liver tissue sampled seven days after combined brain and spinal cord injury. The animals were injected with spin trap components: DETC-Na, iron sulfate, and sodium citrate. The dotted rectangle outlines the region where three lines of the $(DETC)_2$-Fe^{2+}-NO complex are positioned. Arrows denote the hyperfine structure markers of the corresponding complexes.

with NO, which is characterized by an easily recognizable EPR spectrum with g-factor $g = 2.038$ and three hyperfine structure components, is seen in the 330–337 mT field region. The g-factor is specified by well-known formula $h\nu = g\beta B$ [54,55], where the measurement parameters are frequency ν (in the present case, 9.53 GHz) and magnetic field induction B. The g-factor for $(DETC)_2$-Fe^{2+}-NO was determined at the point where the first derivative of the central hyperfine structure (HFS) component intersects the zero line. The EPR spectrum from the Cu^{2+}-$(DETC)_2$ complex with a g-factor of 2.04 is present within the examined magnetic field range. As is known, the spectrum of this complex is split into four HFS components [63,64,71,72].

The average NO content in liver samples was 15.1 ± 4.6 a.u. in the control group and 4.9 ± 1.3 a.u. in the group of injured rats. Figure 2, *a* presents statistical data on the relative integral signal intensities of $(DETC)_2$-Fe^{2+}-NO in the spectra of the studied liver samples. It is evident that the NO production decreased significantly seven days after injury. It is also evident that the measurement data for liver tissues of control animals vary, but the NO content reduction after injury is statistically reliable (t-test, $p = 0.045$). The average Cu content in liver samples was 14.6 ± 11.9 a.u. in the control group and 7.7 ± 3.8 a.u. in the group of injured rats. Figure 2, *b* presents statistical data on the relative integral signal intensities of Cu^{2+}-$(DETC)_2$ in the spectra of the studied liver samples. The copper content decreases by a factor of more than 2; however, owing to the wide scatter of data in control animals, the difference is statistically unreliable.

3. Discussion

Damage to the structure of the brain or spinal cord inflicted by various injuries (strokes, traumas) has catastrophic dysfunctional consequences in the body of humans and animals [73,74]. The damage to neurons and glia is enhanced and prolonged after a stroke or brain injury, since the blood supply to injured tissues is impaired. One of the causes and, in equal measure, consequences of such pathological processes in the brain is hypoxia, which serves as a precursor to many pathological processes in the body [75,76]. One of the unresolved socially important issues is rehabilitation of patients with brain and spinal cord injuries [73,77–79]. The solutions here depend heavily on the current understanding of pathological mechanisms of secondary damage [40,41,80,81]. Dysfunctioning of neural networks induced by spinal cord injury of any etiology (trauma, hemorrhage, tumor or inflammatory processes) is accompanied not only by the loss of central control of somatic and vegetative functions, but also by progressing destructive processes in the nervous tissue [82]. The blood supply to nerve and glial cells and elements of the intercellular matrix is impaired. A cascade of events, which are characterized as „secondary injury“, evolves: in addition to endothelial cell damage and homeostasis disruption, ischemic reperfusion injury triggers full-blown inflammatory processes resulting from the activation of innate immune cells (microglia, oligodendrocytes, and astrocytes) and infiltration of leukocytes (neutrophils and macrophages). These inflammatory cells secrete neurotoxins (proinflammatory cytokines and chemokines, free radicals, excitotoxic amino acids, and NO) that promote the destruction of axons and neurons [81,83]. Such processes alter fatally the relationship between neurons, glial cells, and the intercellular matrix.

Traumatic and ischemic brain injuries are still regarded as one of the most complex problems of modern medicine [16,18,31,74,81]. The study of mechanisms of reparative processes in nervous tissue and the development of new methods for restoration of neuronal structures constitute one of the relevant research directions in physiology and medicine and are of great importance for the development of new therapeutic and rehabilitation strategies [2,18,73,81]. A number of pathological mechanisms contributing to the violation of integrity of nerve and glial cells, the destruction of intercellular matrix, and the disruption of blood vessels are triggered in the case of brain injury and ischemia [84,85]. Similarities in the pathogenesis of these cerebral injuries suggest that treatment strategies protecting against brain tissue ischemia in strokes may also be a viable option for patients with brain injury. Recent studies demonstrate that NO inhalation may have a therapeutic effect [19,86]. It was also found that, under certain conditions, blockade of inducible NO synthase may be therapeutic [87]. Mechanisms of cell injury include glutamate excitotoxicity, oxidative stress, free radical production, apoptosis, and inflammation [74,81,88,89].

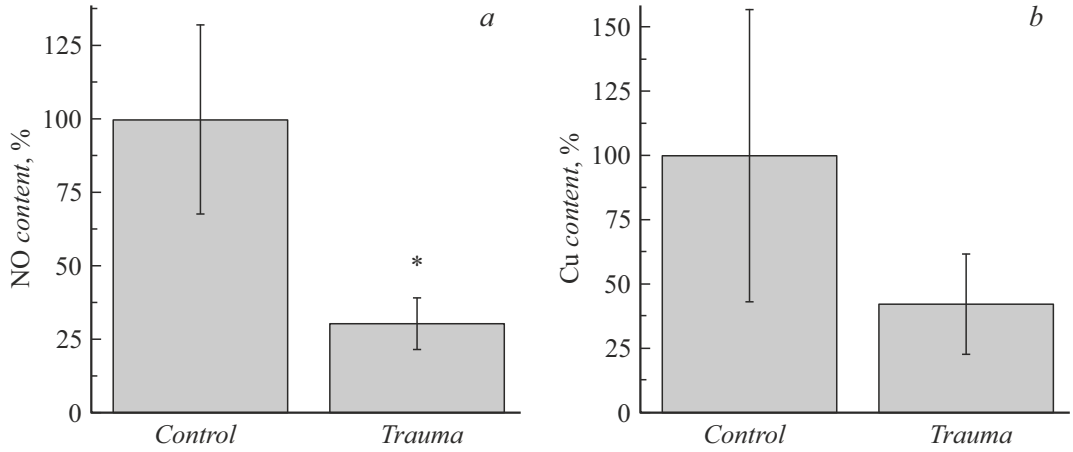

Figure 2. Average NO (*a*) and Cu (*b*) content in liver tissues of control rats and tissues sampled seven days after combined brain and spinal cord injury. The average specific signal intensity of complexes $(DETC)_2$-Fe^{2+}-NO (*a*) and $Cu(DETC)_2$ (*b*) in animal liver tissue samples is plotted (as a percentage of the signal intensity in the control group) on the ordinate axis. * — difference from control (*t*-test, $p < 0.05$).

When a brain injury occurs, both the cells of nervous and glial tissue and tissues of the walls of blood vessels get damaged. An impairment of local blood supply to the damaged brain area is accompanied by intensification of destructive processes, which actually become dominant in the post-traumatic period when the direct influence of the physical factor causing the injury wanes. In turn, lowering of the oxygen levels during tissue hypoxia following injury leads to disruption of the iron redox system, which may catalyze the reaction of formation of free oxygen radicals that cause lipid peroxidation in damaged tissues. In addition, trauma-induced vascular damage results in the release of hemoglobin from red blood cells, giving rise to an additional source of redox-active iron [24,71,91].

There is no doubt that oxidative stress is involved in the mechanisms of pathogenesis in ischemic and traumatic brain injuries [24]. Oxidative stress is a shift in balance between oxidants (ROIs) and antioxidants in favor of the former [25]. ROIs are produced by living organisms in the process of normal cellular metabolism. At low and moderate concentrations, they are involved in physiological processes in the cell; at high concentrations, they cause adverse changes in such cellular components as lipids, proteins, and DNA [12,24,25]. OS contributes to the development of many pathological conditions of various organs [26,92,93]. Therefore, the most important component of cell protection against OS is the antioxidant system [25,27].

Superoxide dismutase is one of the endogenous enzymes that is essential to ROI removal [24,25,93]. SOD is the key enzyme in antiradical protection. There are three isoforms of this enzyme; two of them are Cu, Zn superoxide dismutase, which is the main subtype found in the nervous system [27]. It is known that liver contains the highest amount of this enzyme subtype [94]. Other elements of the antioxidant system, such as catalase, glutathione peroxidase, etc., are also known [27]. Redox-active iron ions are among the main inducers of oxidative stress in tissues [24]. It is also known that copper and iron entering the body through the gastrointestinal tract are transported, stored in liver hepatocytes, and recruited into the blood if certain pathological conditions of tissues, such as ischemia or inflammation, are detected [61]. We have demonstrated a significant reduction in Cu content in the liver of rats with combined brain and spinal cord injury, which may serve as a confirmation of recruitment of copper cations for the synthesis of CuZn superoxide dismutase [46].

Spontaneous restoration of damaged vessels and reperfusion require a certain amount of time. NO and its derivatives play an important role in physiology and pathophysiology of the liver [63,95]. The key functions are performed by endothelial (eNOS) and inducible (iNOS) NO synthases (NOS). eNOS is expressed primarily in endothelial cells of the hepatic artery, portal vein, central vein, and lymphatic vessels. NO derived from eNOS maintains liver homeostasis and suppresses pathological conditions in the liver. In contrast, iNOS is induced in a variety of liver cells, including hepatocytes, Kupffer cells, and other immune cells.

In a number of pathological conditions, iNOS produces large amounts of NO, which is the main source of reactive nitrogen intermediates in this tissue [61,87,95,96]. NO reacts actively with metals, such as Cu and Fe, present in the liver [63.95]. We have demonstrated a significant reduction in NO and Cu content in the liver of rats after combined brain and spinal cord injury, which may be indicative of the development of inflammatory processes in the liver. This conclusion is also supported by the fact that an increased NO concentration has a therapeutic effect in certain liver diseases [97].

The obtained data suggest that combined brain and spinal cord injury is accompanied by a significant suppression of NO production not only in the injured brain area [46], but also in the liver (i.e., an organ that has no direct relation to

the injured part of the body). This proves that changes in blood supply and the transport of various substances in the blood play a prominent part in functioning of the body as a whole.

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Conflict of interest

The authors declare that they have no conflict of interest

Authors' contributions

Kh.L. Gainutdinov and V.A. Kul'chitskii proposed the study and were responsible for the concept, design, coordination, and final approval. Kh.L. Gainutdinov, V.A. Kul'chitskii, V.V. Andrianov, D.I. Silant'eva, and G.G. Yafarova were involved in interpreting the results. V.A. Kul'chitskii, A.V. Nagibov, V.M. Rubakhova, E.V. Fedorova, T.A. Filipovich, and G.G. Yafarova performed modeling of combined brain and spinal cord injury and sampling. L.V. Bazan was responsible for the EPR spectrometer operation. L.V. Bazan, A.I Arslanov, I.B. Deryabina, L.N. Muranova, and D.I. Silant'eva measured the EPR spectra of samples. V.V. Andrianov worked out the procedure for sample processing. V.V. Andrianov and T.Kh. Bogodvid performed calculations and analysis of NO and copper signals in EPR spectra. V.V. Andrianov prepared original figures.

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