DOI 10.1007/s10517-024-06176-y
Bulletin of Experimental Biology and Medicine, Vol. 177, No. 3, July, 2024

GENERAL PATHOLOGY AND PATHOLOGICAL PHYSIOLOGY

Analysis of the Intensity of Nitric Oxide Production in Different Parts of the Spinal Cord after Modeling Combined Cerebral and Spinal Injury

Kh. L. Gainutdinov[1,2], V. A. Kulchitsky[3], G. G. Yafarova[1], V. V. Andrianov[1,2], L. V. Bazan[2], T. K. Bogodvid[1,4], D. I. Silantyeva[1], and A. V. Nagibov[4]

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 177, No. 3, pp. 275-279, March, 2024
Original article submitted November 13, 2023

Using the method of electron paramagnetic resonance spectroscopy, we showed that NO production decreases by 60% ($p<0.05$) in the region located rostral to the spinal cord injury 7 days after combined injury to the brain and spinal cord. At the same time, NO production did not change in the site of spinal cord injury and caudal to the injury. The intensity of NO production in similar parts of the spinal cord in intact animals remained unchanged.

Key Words: *nitric oxide; brain stroke; spinal cord injury; spinal cord; electron paramagnetic resonance*

The discovery of the ability of mammalian cells to synthesize nitric oxide (NO) served as an incentive for studying the role of NO in almost all areas of biology and medicine [1]. It was found that NO is widely spread in the nervous [2], cardiovascular [3], and other systems of the body [1]. The most important function of NO is vasodilation [4]. In the recent years, new facts indicating that changes in biosynthesis of NO and its content in tissues are the main factors in blood flow impairments and the development of hypoxia/ischemia after brain and spinal cord (SC) injuries have appeared [5-10]. Ischemia, hemorrhage, brain or SC injuries are associated with disturbances in metabolic processes and functioning of neurotransmitter systems, including the NO system, which results in the development of phasic pathological processes leading to destruction of nerve and glial cells and extracellular matrix and damage to blood vessels [1,11,12]. The role of NO in pathological processes developing after combined injury to the brain and SC is least studied in this context.

Much scientific attention is attracted to the participation of NO in not only physiological processes, but also in the mechanisms of development of various pathological conditions. NO is involved in mechanisms of secondary injury of the SC [1], when reactive oxygen and nitrogen species initiate secondary damage processes [1]. Duality of the mechanisms of actions of NO is determined by NO content, the severity, nature, and phase of the pathological processes in the brain, and many other factors that require in-depth research [9]. Thus, the NO system is a promising target for therapeutic interventions in the therapy of combined SC and brain injury. The role of NO in the nervous system injury [8,10], in particular, the dynamics of NO

[1]Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, Kazan, Republic of Tatarstan, Russia; [2]Zavoisky Physical-Technical Institute, Federal Research Center Kazan Scientific Center, Russian Academy of Sciences, Kazan, Republic of Tatarstan, Russia; [3]Brain Center, Institute of Physiology, National Academy of Sciences of Belarus, Minsk, Republic of Belarus; [4]Volga Region State University of Physical Culture, Sports, and Tourism, Kazan, Republic of Tatarstan, Russia. ***Address for correspondence:*** kh_gainutdinov@mail.ru. Kh. L. Gainutdinov

production at different stages of SC injuries is little studied [9,13,14]. Presumably, the role of NO in this case depends on the concentration range and the type of cell source and the environment in which it was obtained [10,15].

Electron paramagnetic resonance (EPR) is a modern effective method for the detection and measuring of NO content in biological tissues. A feature of the use of the spin trap technique is the formation of complexes that do not disintegrate at liquid nitrogen temperatures for a long time (days, months), which makes it possible to measure spectra several time after the extraction of biological samples [4,15].

Our aim was to study the intensity of NO production in different parts of the SC after modeling combined injury of the brain and SC by the method of EPR spectroscopy.

MATERIALS AND METHODS

The study was carried out on Wistar male rats (n=10) weighing 200-250 g under ketamine–xylazine–acepromazine anesthesia (55.6 mg/kg ketamine, 5.5 mg/kg xylazine, and 1.1 mg/kg acepromazine, intraperitoneally) [16]. The same mixture in similar dosages was used in vagotomy, the duration of which was shorter than the simulation of injury in the brain and SC. The advantage of this mixture is deep anesthesia and rapid recovery of laboratory animals from anesthesia [16]. The rats were housed in standard vivarium conditions with unrestricted access to water and food. Intact animal served as controls (n=10).

Combined brain and SC injury was modeled at the Brain Center, Institute of Physiology, National Academy of Sciences of Belarus, according to the protocol approved by the Ethics Commission of the Institute of Physiology, National Academy of Sciences of Belarus (Protocol No. 1 of January 31, 2019). Anesthetized laboratory rats were fixed on a surgical table in a prone position using stretchers on the limbs. The head was fixed; after depilation, the skin was treated with a 2% iodine solution and the skin and soft tissues were cut (10-12 mm) along the midline with a scalpel. The periosteum was locally removed in the projection of the precentral gyrus, then craniotomy was performed with a dentist drill followed by local destruction of the brain tissue in the precentral region of the left hemisphere with a stylet (the operation takes 3-4 min). Then, the wound was sutured and the skin was treated with a 2% iodine solution. At the next stage, the operation was continued at the lumbar level of the SC. The skin was depilated and treated with 2% iodine solution, an incision of the skin and soft tissues was made in the projection of the lumbar vertebra. Then the stylet was introduced into the SC at the level of the first lumbar vertebra (L1); the duration of bleeding from the wound after removal of the stylet was recorded. After the bleeding stopped, the wound was sutured with two stitches and the skin was treated with a 2% iodine solution.

One week after surgery, SC samples at the site of injury and rostral and caudal from site of injury were isolated from all animals (a total of 30 SC fragments). The site of injury was localized and visualized by the remaining trace of hemorrhage. Rostral to the site of injury, a rostral tissue sample of the same size as injured was identified. Caudal to the site of injury, a caudal fragment of the same size was identified. Tissue samples were frozen in liquid nitrogen immediately after extraction and stored in a refrigerator at low temperature until assay. The weight of the samples was about 100 mg.

The intensity of NO production was measured by EPR spectroscopy at the Zavoisky Physical-Technical Institute of the Federal Research Center Kazan Scientific Center of the Russian Academy of Sciences. The spin trap technique was used [17]. As in previous experiments [10], the components of the spin trap for NO (diethyldithiocarbamate of sodium (DETC-Na), $FeSO_4$, and sodium citrate) were injected 30 min before tissue sampling. DETC-Na was administered intraperitoneally at a dose of 500 mg/kg in 2.5 ml of water, a mixture of ferrous sulfate ($FeSO_4 \times 7H_2O$; Sigma) at a dose of 37.5 mg/kg and sodium citrate at a dose of 187.5 mg/kg prepared immediately before injection was administered subcutaneously at 3 points: the right and left thighs and in the rostral part of the interscapular region [10,13]. These components form a ternary complex DETC-Fe^{2+} that binds NO yielding a stable radical $(DETC)_2$-Fe^{2+}-NO. Paramagnetic complex $(DETC)_2$-Fe^{2+}-NO is characterized by an easily recognizable EPR spectrum with a g-factor value of 2.038 and 3 hyperfine structure components [17,18].

The spectra of $(DETC)_2$-Fe^{2+}-NO complex of the biological samples were recorded on a Bruker X-band (9.5320 GHz) EMX/plus spectrometer. The sample was placed in a two-cavity double resonator (model ER 4105DR, Bruker) at a magnetic field modulation frequency of 100 kHz, modulation amplitude of 2 G, microwave radiation power of 2 mW, time constant of 327 msec, and temperature of 77 K. The modulation amplitude, gain, and microwave power in all experiments were selected with the condition that there was no overmodulation and saturation of the EPR signal and remained the same throughout all measurements. The amplitude of the EPR spectra was consistently normalized to the sample weight to ensure accurate comparison and analysis [10].

The obtained results were processed using SigmaPlot 11.3 software. The significance of differences

was assessed using the Student's *t* test after testing for normality of distribution and equality of variances. The data are presented as the mean±standard error of the mean (*M±SEM*). The differences were considered statistically significant at $p<0.05$.

RESULTS

EPR spectroscopy was employed to study the relative intensity of NO production in different parts of the SC (the site of injury and fragments rostral and caudal to the site of injury) after combined injury to the brain and SC. The EPR spectra of SC tissue samples from control and injured rats 7 days after combined brain and SC injury are presented in Figure 1. The signals from $(DETC)_2$-Fe^{2+}-NO complexes were shown with amplitudes equal to their contribution to the spectrum of the sample.

A comparative analysis of the relative NO content in different SC parts revealed no significant differences. The data on the integral intensity of the signal $(DETC)_2$-Fe^{2+}-NO in the spectra of the studied samples are presented in Figure 2. Seven days after the combined injury, NO production insignificantly decreased in the site of injury and remained unchanged in the area below the site of injury. At the same time, NO production in the area above the site of injury significantly decreased by 60% ($p<0.05$). The intensity of NO production in intact animals remained unchanged. Thus, combined injury to the brain and SC was accompanied by a decrease in NO production in the area located above the site of the injury.

Traumatic and ischemic injuries to the brain and SC remain the most difficult problem of modern medicine [6,13,19]. In addition to recovery processes, many pathological mechanisms that contribute to the disruption of the integrity of nerve and glial cells, as well as damage to blood vessels, are realized in case of injury and ischemia of the brain and SC [11]. Secondary SC injury occurs as a result of a cascade of biochemical and cellular processes that are triggered by primary processes, which exacerbates tissue damage and limits repair processes. The results of our experiments and published data prove that NO often plays a trigger role in these processes [6,16]. However, the dynamics of NO production and the possibility of using NO donors or NO synthase blockers remain controversial [14]. The data obtained in our experiments showed that in combined trauma of the brain and SC, NO content changes in different ways in areas rostral and caudal to site of injury, and again attests to the important role of both local and central regulation in the recovery processes of the nervous system after damage. These factors should be taken into account when creating and implementing effective therapeutic and rehabilitation strategies for SC pathology [20].

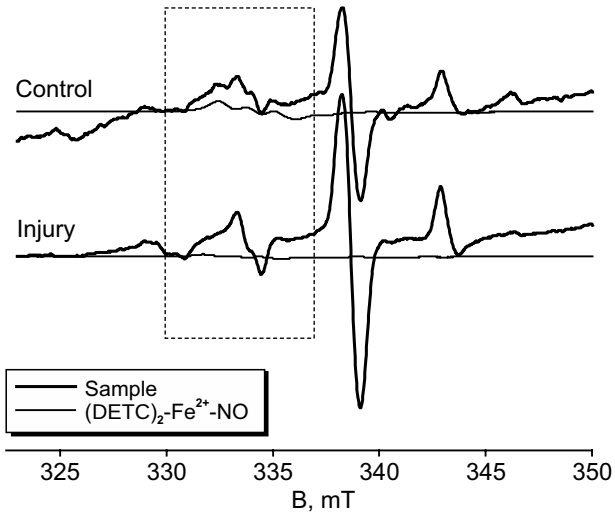

Fig. 1. Representative EPR spectra of SC tissue samples from the area above the site of SC injury in the control and 7 days after combined injury to the brain and SC. Heavy line is the spectrum of the sample, the thin line is the signal from the $(DETC)_2$-Fe^{2+}-NO complex. The frame shows the magnetic field area $(DETC)_2$-Fe^{2+}-NO complex. Temperature is 77 K.

Author contributions. Kh. L. Gainutdinov and V. A. Kulchitsky conceived of the study and were responsible for the conception, design, and coordination of the research. V. A. Kulchitsky and A. V. Nagibov carried out the modeling of combined injury of the brain and spinal cord. G. G. Yafarova and A. V. Nagibov made extraction of tissue samples. L. V. Bazan and D. I. Silantyeva carried out measurements of the EPR spectrum of samples. V. V. Andrianov and

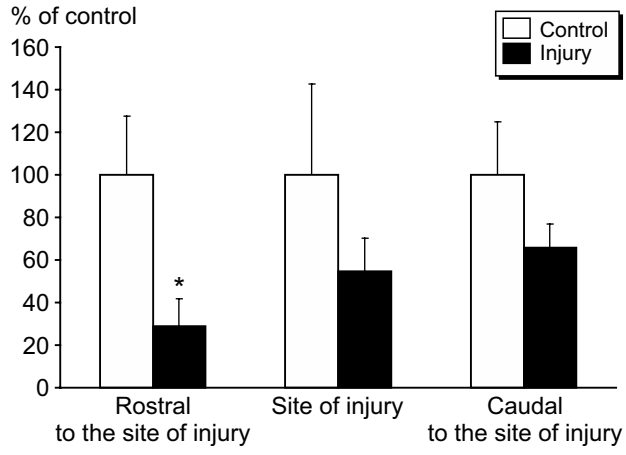

Fig. 2. Changes in NO content in different areas of SC 7 days after combined injury to the brain and SC. *$p<0.05$ in comparison with the control. Ordinate: mean integral intensity of $(DETC)_2$-Fe^{2+}-NO complex in spectra of the samples (as a percentage relative to the content in the corresponding part of the SC of the control group).

T. K. Bogodvid carried out analysis of NO signals in EPR spectra. Kh. L. Gainutdinov, V. V. Andrianov, G. G. Yafarova, T. K. Bogodvid, V. A. Kulchitsky, and D. I. Silantyeva analysed the literature data, wrote and proofreaded the paper. V. V. Andrianov created the original figures.

The modeling of injuries to the brain and spinal cord was conducted at the Brain Center, Institute of Physiology, National Academy of Sciences of Belarus and was supported by the Belarusian Republican Foundation for Basic Research (grant M23RNF-067). The measurements of EPR spectra and data processing were performed at the Zavoisky Physical-Technical Institute of the Federal Research Center Kazan Scientific Center of the Russian Academy of Sciences and was supproted by the Russian Science Foundation (grant No. 23-45-10004). Part of the data processing were performed at the Kazan Federal University supported by the Strategic Academic Leadership Program of Kazan Federal University (PRIORITY-2030).

Conflict of interest. The authors have no conflicts of interest to declare.

REFERENCES

1. Lundberg JO, Weitzberg E. Nitric oxide signaling in health and disease. Cell. 2022;185(16):2853-2878. doi: 10.1016/j.cell.2022.06.010
2. Steinert JR, Chernova T, Forsythe ID. Nitric oxide signaling in brain function, dysfunction, and dementia. Neuroscientist. 2010;16(4):435-452. doi: 10.1177/1073858410366481
3. Reutov VP, Okhotin VE, Shuklin AV, Sorokina EG, Kositsyn NS, Gurin VN. Nitric oxide (NO) and NO cycle in myocardium: molecular, biochemical and physiological aspects. Usp. Fiziol. Nauk. 2007;38(4):39-58. Russian.
4. Vanin AF. Dinitrosyl iron complexes with thiol-containing ligands as a base for developing drugs with diverse therapeutic activities: Physicochemical and biological substantiation. Biophysics. 2017;62(4):509-531. doi: 10.1134/S0006350917040224
5. Manukhina EB, Tseilikman VE, Karpenko MN, Pestereva NS, Tseilikman OB, Komelkova MV, Kondashevskaya MV, Goryacheva AV, Lapshin MS, Platkovskii PO, Sarapultsev AP, Alliluev AV, Downey HF. Intermittent hypoxic conditioning alleviates post-traumatic stress disorder-induced damage and dysfunction of rat visceral organs and brain. Int. J. Mol. Sci. 2020;21(1):345. doi: 10.3390/ijms21010345
6. Wierońska JM, Cieślik P, Kalinowski L. Nitric oxide-dependent pathways as critical factors in the consequences and recovery after brain ischemic hypoxia. Biomolecules. 2021;11(8):1097. doi: 10.3390/biom11081097
7. Wang Y, Hong F, Yang S. Roles of nitric oxide in brain ischemia and reperfusion. Int. J. Mol. Sci. 2022;23(8):4243. doi: 10.3390/ijms23084243
8. Garry PS, Ezra M, Rowland MJ, Westbrook J, Pattinson KT. The role of the nitric oxide pathway in brain injury and its treatment – from bench to bedside. Exp. Neurol. 2015;263:235-243. doi: 10.1016/j.expneurol.2014.10.017
9. Yafarova GG, Andrianov VV, Yagudin RKh, Shaikhytdinov II, Gainutdinov KhL. Effects of NO synthase blocker L-NAME on functional state of the neuromotor system during traumatic disease of the spinal cord. Bull. Exp. Biol. Med. 2017;162(3):316-319. doi: 10.1007/s10517-017-3604-6
10. Andrianov VV, Kulchitsky VA, Yafarova GG, Zamaro AS, Tokalchik YP, Bazan LV, Bogodvid TK, Iyudin VS, Pashkevich SG, Dosina MO, Gainutdinov KL. Comparative study of the intensity of nitric oxide production and copper content in hippocampus of rats after modeling of hemorrhagic stroke and brain injury. Appl. Magn. Reson. 2021;52:1657-669. doi: 10.1007/s00723-021-01423-1
11. Guo ZN, Shao A, Tong LS, Sun W, Liu J, Yang Y. The role of nitric oxide and sympathetic control in cerebral autoregulation in the setting of subarachnoid hemorrhage and traumatic brain injury. Mol. Neurobiol. 2016;53(6):3606-3615. doi: 10.1007/s12035-015-9308-x
12. Chen ZQ, Mou RT, Feng DX, Wang Z, Chen G. The role of nitric oxide in stroke. Med. Gas Res. 2017;7(3):194-203. doi: 10.4103/2045-9912.215750
13. Maggio DM, Singh A, Iorgulescu JB, Bleicher DH, Ghosh M, Lopez MM, Tuesta LM, Flora G, Dietrich WD, Pearse DD. Identifying the long-term role of inducible nitric oxide synthase after contusive spinal cord injury using a transgenic mouse model. Int. J. Mol. Sci. 2017;18(2):245. doi: 10.3390/ijms18020245
14. Tardivo V, Crobeddu E, Pilloni G, Fontanella M, Spena G, Panciani PP, Berjano P, Ajello M, Bozzaro M, Agnoletti A, Altieri R, Fiumefreddo A, Zenga F, Ducati A, Garbossa D. Say “no” to spinal cord injury: is nitric oxide an option for therapeutic strategies? Int. J. Neurosci. 2015;125(2):81-90. doi: 10.3109/00207454.2014.908877
15. Deryagin OG, Gavrilova SA, Gainutdinov KL, Golubeva AV, Andrianov VV, Yafarova GG, Buravkov SV, Koshelev VB. Molecular bases of brain preconditioning. Front. Neurosci. 2017;11:427. doi: 10.3389/fnins.2017.00427
16. Ivanov AI, Kulchitsky VA, Romanovsky AA. Role for the cholecystokinin-A receptor in fever: a study of a mutant rat strain and a pharmacological analysis. J. Physiol. 2003;547(Pt 3):941-949. doi: 10.1113/jphysiol.2002.033183
17. Mikoyan VD, Burgova EN, Borodulin RR, Vanin AF. Dinitrosyl iron complexes with thiol-containing ligands exist in living organisms mainly in the binuclear form. Biophysics. 2020;65(6):972-980. doi: 10.1134/S0006350920060111
18. Jakubowska MA, Pyka J, Michalczyk-Wetula D, Baczyński K, Cieśla M, Susz A, Ferdek PE, Płonka BK, Fiedor L, Płonka PM. Electron paramagnetic resonance spectroscopy reveals alterations in the redox state of endogenous copper and iron complexes in photodynamic stress-induced ischemic mouse liver. Redox Biol. 2020;34:101566. doi: 10.1016/j.redox.2020.101566
19. Che X, Fang Y, Si X, Wang J, Hu X, Reis C, Chen S. The role of gaseous molecules in traumatic brain injury: an updated review. Front. Neurosci. 2018;12:392. doi: 10.3389/fnins.2018.00392
20. Chari A, Hentall ID, Papadopoulos MC, Pereira EA. Surgical neurostimulation for spinal cord injury. Brain Sci. 2017;7(2):18. doi: 10.3390/brainsci7020018