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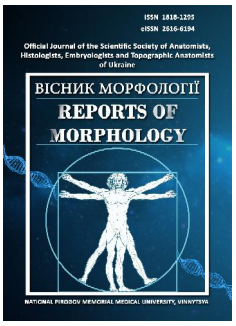

Changes of rat's brain vesseles after air shock wave exposure

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CONFLICT OF INTEREST

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Mild blast-induced traumatic brain injury is common among the military, resulting in cognitive impairment, reduced socialization, which leads to disability and, as a result, a deterioration in the quality of life. It is considered that blood-brain barrier disruption and microvascular dysfunction are the key to this type of injury. The purpose of study was to study changes in brain vessels after air shock wave exposure. The study was carried out on 48 mature male Wistar rats, which were randomly divided into 2 groups: an experimental group, in which animals were subjected to inhalation anesthesia using halothane and exposed to a shock wave with an overpressure of 26.4±3.6 kPa, and a Sham group. After simulation of injury on days 1st, 3rd, 7th, 14th, and 21st, the rats were euthanized and the brain was removed and after all subjected to standard histological procedures and stained with hematoxylin and eosin. For immunohistochemical studies, as primary antibodies were used eNOS. The finished preparations were examined by light microscopy and photographed. Disorders of the cerebral vessels in experimental rats were detected from day 1st of the posttraumatic period. It was found that the blast wave led to vascular rupture, as well as increased vascular permeability with diapedesis of red blood cells and cerebral edema for up to 21st days. Focal violations of the vascular wall integrity in cortical and hippocampal hemocapillaries, venular link of the submembrane vessels; changes in the morphology of the metabolic vessels endothelium; uneven blood filling of the brain vessels were of major importance. These changes indicate that increased eNOS expression leads to dilation of cerebral vessels, which is a compensatory mechanism in response to injury to improve cerebral blood circulation. However, eNOS is not involved in vasodilation, which we observed up to 21st day post-trauma.

Key words: *blood-brain barrier, explosion, trauma, brain, blood vessels.*

Introduction

In the context of military operations, terrorist attacks or man-made disasters, the number of injuries caused by explosions is increasing [18, 22]. Depending on many factors, including the body position and distance from the explosion, different organs injuries occur with varying degrees of severity. The most sensitive to the blast wave are lungs, eardrum and brain [7]. Mild blast-induced traumatic brain injury is a common phenomenon among military, leading to cognitive impairment, reduced socialization and disability, as well as a deterioration in quality of life [9]. Therefore, in order to improve medical and social assistance to the victims of the explosion, it is necessary to find out the mechanisms of brain damage.

Despite the fact that the exact pathophysiological mechanisms of blast injuries are not yet fully understood, it is known that the blast wave is the main pathogenic factor in such injuries [11]. Numerous studies show that when a blast wave hits the head, part of the energy is reflected from the skull bones, and the part that passes through the tissues extremely excites the phonon continuum, which leads to brain cells damage at the subcellular level [24]. After the primary injury, a wide range of pathobiochemical reactions of secondary alteration are triggered, including the release of pro- and inflammatory mediators, the development of oxidative stress, etc. [19, 32]. Many histopathological studies clearly indicate a blood-brain barrier disorder [14]. Is considered that blood-brain barrier disruption and microvascular dysfunction are key factors in the course of blast-induced traumatic brain injury and further development of neurodegeneration [5, 30]. However, studies of these changes pathogenesis are multidirectional, which does not provide a holistic picture and requires further research on this issue. A well-known factor in the regulation of cerebral blood vessels is eNOS,

the increase or decrease of which lead to vascular tone and permeability changes [10]. Lots of studies on eNOS in cerebrovascular disorders have shown that eNOS deficiency leads to dysregulation of systemic blood pressure and a decrease of the blood-brain barrier protective function [6, 25]. While the increase in eNOS content performs a protective function by improving the removal of toxic substances and excess fluid from the brain [1].

The purpose of the study is to investigate changes in brain vessels after exposure by an air shock wave.

Materials and methods

The work was carried out as the framework of the planned initiative research topic of the Pathological Anatomy, Forensic Medicine and Pathological Physiology Department of Dnipro State Medical University "Mechanisms of changes formation in the central nervous system after exposure to extreme factors", state registration number 0120U105394.

The study was carried out on 48 sexually mature male Wistar rats weighing 220-270 g, aged 6-7 months. The animals were kept in standard conditions and on a standard diet at the vivarium of the Dnipro State Medical University. All studies were conducted in accordance with modern international requirements and standards for the humane treatment of animals (Council of Europe Convention of 18.03.1986 (Strasbourg), the 1975 Helsinki Declaration, revised and supplemented in 2000, Law of Ukraine, February 21, 2006 No. 3447-IV), as evidenced by an extract from the meeting of the Biomedical Ethics Commission of Dnipro State Medical University minutes No. 3, November 2, 2021.

The selected rats were randomly divided into two groups: group I - experimental (n=24), animals of which were subjected to inhalation anesthesia with halothane (Halothan Hoechst AG, Germany), fixed in a horizontal position on the abdomen with the head to the muzzle cut at a distance of 5 cm and simulated blast brain injury by generating a shock wave with an overpressure of 26.4± 3.6 kPa on a self-made device (Ukraine patent № 146858). The overpressure was measured using an electronic manometer BIT02B-10B (AEP transducers, Italy). And group II - sham (n=24). After simulation of mild blast-induced traumatic brain injury on 1st, 3rd, 7th, 14th, and 21st days, rats were euthanized with a lethal dose of halothane, and then brains were removed. Subsequently, the material was fixed in a 10 % formalin solution (pH 7.4) for at least 24 hours at room temperature [27]. The infiltration process was performed on a Microm STP-120 histoprocessor (Thermo Fisher Scientific, Germany). The samples were dehydrated in increasing concentrations of isopropanol (70 %, 80 %, 95 %, and in three steps in 100 % for 90 min per step 1). After that, the tissues were embedded in paraffin blocks using a HistoStar embedding station (Thermo Fisher Scientific, USA). Serial sections of no more than 4 μm thickness were obtained from the blocks using a Thermo HM 355S microtome (Thermo Fisher Scientific, Germany). Sections of each tissue sample were used for general histological tissues staining with hematoxylin and eosin [27].

For immunohistochemical study, 4 μm thick sections were applied to Superfrost adhesive slides (Thermo, Germany), then they were deparaffinized with xylene and dehydrated. The activity of endogenous peroxidase was blocked with a 3 % solution of hydrogen peroxide in 70 % methanol for 20 min at room temperature.

Then the sections were washed in three steps with sodium phosphate buffer followed by heat-induced antigen retrieval (HIAR) by heating in a water bath in citrate buffer with pH 6.0 or tris-EDTA buffer with pH 9.0 (20 minutes after reaching a temperature of 98°C) with symmetrical arrangement of slides in the cuvette with the addition of 2 ml of Triton-X100 detergent (Sigma, Germany) per 200 ml of buffer [27].

After washing in three steps with sodium phosphate buffer, the slides were placed on a moistened plate and incubated with a 1 % blocking serum solution (normal goat serum) in 1 % BSA (bovine serum albumin) for 20 minutes. Antibodies to endothelial NO synthase, eNOS (clone M221, dilution 1:400, Abcam, UK) were used as primary antibodies.

Sections were incubated with primary antibodies in a humid chamber at 40 0C overnight. Visualization was performed using the Master Polymer Plus Detection System reagent kit (Master Diagnostica, Spain), which was completed by the reaction of DAB chromogen with hydrogen peroxide in the presence of horseradish peroxidase with the formation of a brown color at the sites of binding of diagnostic antibodies to test markers [27]. For the nuclei background staining, sections were additionally stained with Gill's hematoxylin for 30 seconds. Then they were dehydrated in increasing concentrations of alcohol, cleared in xylene, and placed in the final medium under a coverslip.

Microscopy of histological specimens was performed using an Axio Imager 2 microscope (Zeiss, Germany) at x100 and x200 magnification.

Results

Brain cells have a high level of metabolic processes [17], which requires adequate arterial blood supply and venous outflow (Fig. 1).

The cerebral vessels network is strictly regulate the penetration of substances from the vessels into the brain and ensures the outflow of metabolic products [9]. Disorders of cerebral vessels, the wall of which is a component of the blood-brain barrier, were observed in experimental rats throughout the study period starting from 1st day (Fig. 2).

Among the changes that occurred in the brain vessels after exposure to an air shock wave during the posttraumatic period, the leading role was played by focal violations of the vascular wall integrity in cortical and hippocampal hemocapillaries, the venular link of the subcortical vessels;

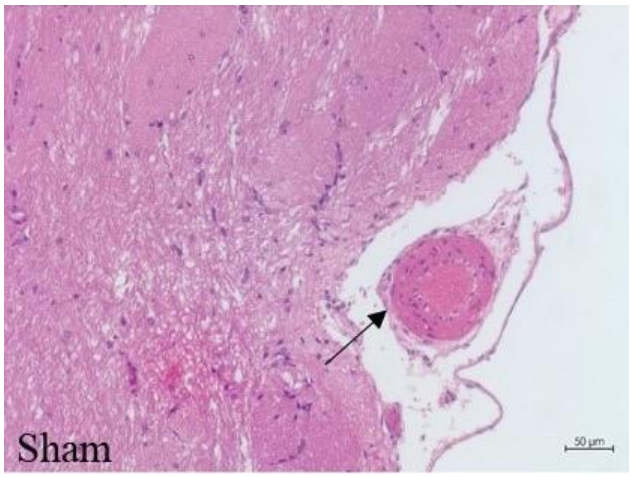

Fig. 1. Cross histological section of the subclavian subpial artery of the sham rat brain. The arrow points to the cerebral vessel. Staining: hematoxylin and eosin, magnification x200.

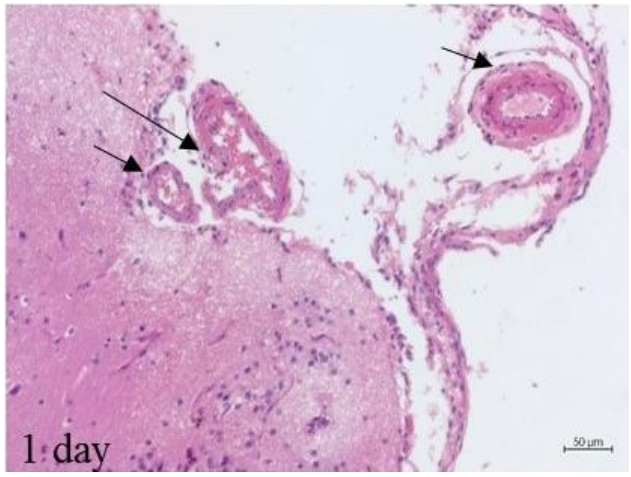

Fig. 2. Subpial vessels of the experimental rat brain (1st day). The arrow points to the cerebral vessels that underwent changes due to the blast wave. Venule wall rupture. Staining: hematoxylin and eosin, magnification x200.

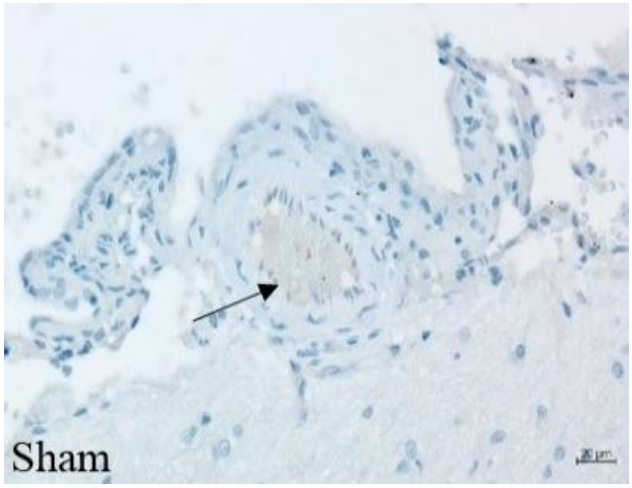

Fig. 3. Expression of eNOS in brain vessels of sham rats. The arrow indicates a brain vessel with low eNOS expression. Magnification x200.

changes in the morphology of the metabolic vessels endothelium; and uneven blood filling of the brain vessels. Violation of the vessel wall integrity was accompanied by the appearance of diapedesis and small-focal hemorrhages, neutrophil release and formation of perivascular infiltrates of polymorphonuclear cells on 1st day (see Fig. 2) and persisted up to 7th day in the group of experimental animals. By the end of the third week of the experiment, we recorded uneven blood filling of the microcirculatory system vessels on histological preparations of the brain, which was due to impaired hemocapillary patency due to endothelial cell edema and impaired rheological properties of blood with the formation of stasis and aggregation of blood cells. Also, the patency of the hemocapillary bed was affected by perivascular edema, which increased in the first week of the posttraumatic period.

Due to the observed impairment of blood-brain barrier permeability, vascular wall integrity, and regional blood

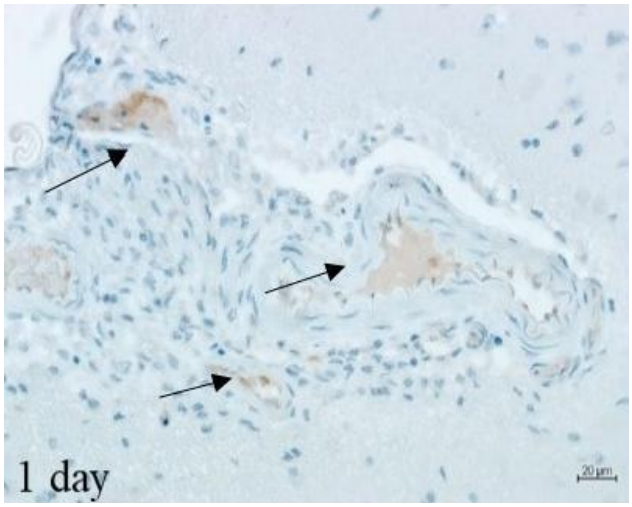

Fig. 4. Expression of eNOS in the brain vessels of the experimental group rats on 1st day of the posttraumatic period. Arrow indicates brain vessels with moderate eNOS expression. Magnification x200.

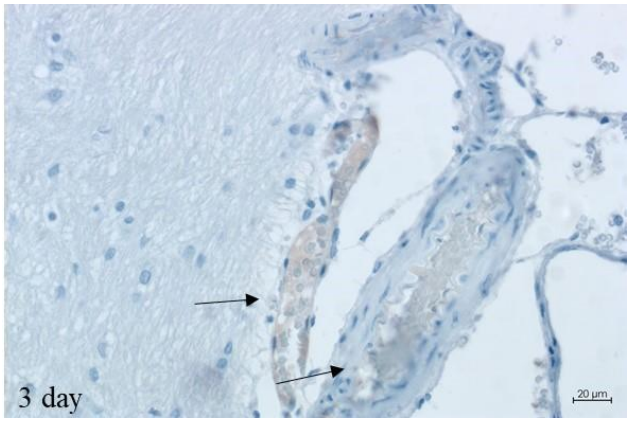

Fig. 5. Expression of eNOS in the brain vessels of the experimental group rats on 3rd day of the posttraumatic period. The arrow indicates the brain vessels with eNOS expression. Magnification x200.

supply in rats with mild blast-induced traumatic brain injury, it was decided to investigate the degree of eNOS expression as the hypoxia and ischemia marker.

A slight expression of eNOS was detected in the samples of the sham group rats (Fig. 3).

Comparison of the results of immunohistochemical detection of eNOS in the brain vessels showed the presence of this enzyme expression in the endothelium of the brain vessels of experimental rats on 1st day (Fig. 4) and 3rd day (Fig. 5).

The shape of the vessels, in the endothelial cells of which we noted the expression of eNOS, was corrugated, and some vessels were paretic dilated or collapsed (see Figs. 4, 5).

In the dynamics of the further course of the posttraumatic period, the degree of eNOS expression in the brain vessels of the experimental group rats did not differ from that of the sham group.

Discussion

The studied and established consequences of mild blast-induced traumatic brain injury in the acute period are disruption of the blood-brain barrier, which leads to energy and metabolic changes, as well as cerebral edema [3]. Most scientists thought that these changes underlie the cognitive functions of the brain and the subsequent development of neurodegeneration [18]. In the case of mild blast-induced traumatic brain injury, which was simulated using our own device, we found vascular wall disorders with diapedesis hemorrhages, the nonruptured vessels wall edema, and cerebral edema resulting from blood-brain barrier dysfunction. Such changes in blood-brain barrier vessels are associated with direct compressive shock wave loading and indirect cavitation, which together lead to overstretching and detachment of endothelial cell membranes [30].

We also found the brain itself diffuse damage, which causes an inflammatory process involving neutrophils. Comparing our results with previous studies which are showing an existing systemic cytokine response to mild blast-induced traumatic brain injury, we think that an active inflammatory response is unfolding [21, 23, 27]. All of these changes, both individually and in combination, require the launch of compensatory mechanisms to maintain the brain functional activity [13]. However, the mechanisms of both blood-brain barrier dysfunction and recovery have not been thoroughly investigated. Thus, there is a need for their in-depth study.

Vascular tone ensures adequate blood supply of the brain, the regulation of which is complex and clearly controlled by the nervous and humoral systems. Among the numerous regulatory systems, it is important to understand the function of the endothelium, which affects the tone of the brain vessels both chemically and through interaction with neurons. This complex allows not only to regulate vascular tone, but also to control the permeability of the blood-brain barrier [2, 16].

Among the chemical factors involved in the regulation of cerebral vascular tone, eNOS is known to be involved in brain protection during various acute cerebrovascular disorders, including traumatic brain injuries of various genesis [8]. Endothelial nitric oxide synthase is a membrane-binding enzyme and the main source of NO, which is a secondary messenger of guanylyl cyclase-related receptor activation in the central nervous system and is involved in autoregulation of cerebral blood flow and regulation of neuronal plasticity [15].

Many literature sources have shown that increased eNOS activity is a protective mechanism that is realized in response to, at least, the synthesis of pro- and inflammatory cytokines, the development of oxidative stress [24, 26, 30]. After all, NO, which is an autocrine and paracrine signaling factor, leads to dilatation of the brain arterial vessels and also has antithrombotic effects. These properties help to improve the brain blood supply [28, 31]. However, there is evidence to suggest a negative effect of increased eNOS activity. Nevertheless, eNOS promotes vasodilation and increased permeability, which leads to the entry of pro- and inflammatory mediators into the brain, which increases inflammation itself [20].

We consider that eNOS activation occurs when blood-brain barrier vessels are disrupted and it is one of the compensatory mechanisms in response to primary and secondary damage, in particular, to the development of oxidative stress. It is known that mild blast-induced traumatic brain injury leads to mitochondria primary damage and, as a result, free radicals are formed. Subsequently, due to blood-brain barrier damage, hypoxia occurs, which also contributes to free radicals formation. Taken together, oxidative stress itself leads to endothelial dysfunction and eNOS activation [20]. After all, NO is known to have antioxidant properties due to its ability to reduce the formation of superoxide anion by activating superoxide dismutase [12]. However, as our study showed, this occurs only on 1st and 3rd days after simulation of mild blast-induced traumatic brain injury. Next, obviously, in the pathogenesis of vascular wall dilation involve other mechanisms that need to be further investigated.

Conclusions

1. As a result of the blast wave exposure disorders of the cerebral vessels were detected.

2. An increase in eNOS expression in the cerebral vessels of rats with mild blast-induced traumatic brain injury on 1st and 3rd days of the posttraumatic period was found in comparison with sham rats.

3. These changes indicate that increased eNOS expression leads to cerebral vasodilation, which is a compensatory mechanism in trauma response to improve cerebral circulation. However, eNOS is not involved in vasodilation, which we observed up to 21st days post-trauma.

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ЗМІНИ СУДИН ГОЛОВНОГО МОЗКУ ЩУРІВ ПІСЛЯ ДІЇ ПОВІТРЯНОЇ УДАРНОЇ ХВИЛІ

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Поширеною серед військових є легка вибухо-індукована травма головного мозку, наслідками якої є когнітивні порушення та зниження соціалізації людини, що призводить до втрати працездатності і, в результаті, до погіршення якості життя. Вважається, що саме порушення гематоенцефалічного бар'єру та мікросудинна дисфункція є ключовими у перебігу даного виду травми. Метою роботи стало вивчення змін в судинах головного мозку після впливу повітряної ударної хвилі. Дослідження проведено на 48 статевозрілих щурах самцях лінії Wistar, які випадковим чином були розділені на 2 групи: експериментальну (тварин піддавали інгаляційній анестезії із застосуванням галотану та впливу ударної хвилі з надлишковим тиском 26,4±3,6 кПа) і контрольну. Після відтворення травми на 1, 3, 7, 14 та 21 добу щурів піддавали евтаназії з наступним вилученням головного мозку, котрий у подальшому піддавали стандартним гістологічним процедурам. Гістологічні зрізи забарвлювали гематоксиліном та еозином. Для імуногістохімічного дослідження у якості первинних використовували антитіла до eNOS. Готові препарати досліджували за допомогою світлової мікроскопії та фіксували фотоапаратом. Виявлено порушення судин головного мозку у щурів експериментальної групи з 1 доби посттравматичного періоду. Встановлено, що вибухова хвиля призвела до розриву судин, а також підвищення судинної проникності з діапедезною екстравазацією еритроцитів і набряком головного мозку до 21 доби. Провідне значення мали фокальні порушення цілісності судинної стінки в кортикальних та гіпокампальних гемокапілярах, у венулярній ланці підоболонкових судин; зміни морфології ендотелію обмінних судин; нерівномірне кровонаповнення судин мозку. Порівняння отриманих результатів імуногістохімічного визначення eNOS в судинах головного мозку показало наявність експресії цього ензима в ендотелії судин головного мозку експериментальних щурів у 1 та 3 добу. Таким чином, встановлені нами зміни свідчать про те, що підвищення експресії eNOS призводить до розширення судин головного мозку, що є компенсаторним механізмом у відповідь на травму для покращення кровообігу головного мозку. Проте eNOS не бере участь у розширенні судин, яке ми спостерігали до 21 доби посттравматичного періоду.

Ключові слова: *гематоенцефалічний бар'єр, вибух, травма, головний мозок, кровоносні судини.*

Author's contribution:

Kozlova Yu. V. - conceptualization, providing equipment for the experiment, supervision.
Kozlov S. V. - data visualization, conducting an experiment, methodology and writing an original draft.
Maslak H. S. - project administration.
Bondarenko O. O. - formal analysis and verification, review writing and editing.
Dunaev O. V. - software for morphometric research, histological analysis.
Oberemok M. H. - resources.