The state of antioxidant system and emotional status in rats with mild blast-induced traumatic brain injury

Yu. V. Kozlova, H. S. Maslak, O. V. Netronina, O. Y. Abramova, S. V. Kozlov

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

The aim was to evaluate the markers of emotional status and the antioxidant system activity in rats with blast-induced traumatic brain injury.

Materials and methods. The study carried out on 85 sexually mature male Wistar rats in compliance with the current legislation on humane treatment of animals. The selected rats were randomly divided into three groups: I – study in the Barnes maze; II – study in the Open Field test; and III – study of glutathione reductase activity in a solution of rat erythrocyte’s hemolysate. In each of these groups, three groups were formed: Experimental – exposed to a shock wave with an overpressure of 26.4 ± 3.6 kPa; the Sham – subjected only to halothane and fixation; and the Intact group.

Results. Showed a disturbance in the emotional status and the oxidative stress development in rats with blast-induced trauma. The time of experimental rats freezing in the Barnes maze increased by 69 % (p < 0.01) on 28th day compared to 1st day, indicating the development of fear and negative emotionality. Changes in the defecation acts number indicate the development of anxiety in the acute period (1–3 days), followed by a depression-like state. The glutathione reductase activity in experimental rats was significantly higher compared to sham and intact rats, but gradually decreased inside in the experimental group. The results of the correlation analysis indicate the presence of weak relationships between the freezing time and the glutathione reductase activity. The positive relationship of a strong degree was found between the number of defecation acts and glutathione reductase on 1st day and a negative relationship of a strong degree was found on 7th day.

Conclusions. We suppose that oxidative stress is a link, but not the leading one, in the pathogenesis of emotional disturbance in rats with blast-induced brain injury.

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skull bending dynamics, brain impact due to acceleration, and the formation of water bubbles (cavitation), which also cause damage [1]. Whereas TBI is caused by a localized effect of a mechanical factor, resulting in hemorrhage [5].

After the primary damage by the BW, a pathobioc hemical reactions cascade is triggered, which are factors of secondary alteration, among which oxidative stress (OS) plays a leading role [6]. Modern studies of a wide range of antioxidant system markers in TBI prove that OS has an extremely negative effect on the brain both in the acute period, at least due to lipid peroxidation of neuronal membranes, and has consequences in the long-term posttraumatic period, when, due to metabolic changes, neurodegeneration is observed [6,7,8].

However, the association of OS with emotional status changes in the acute period of mild bTBI has not been established.

Aim

Therefore, the aim of the current study was to evaluate the markers of emotional status and the antioxidant system activity in rats with blast-induced traumatic brain injury.

Materials and methods

The study was carried out on 85 sexually mature male Wistar rats, weight 220–270 g, aged 6–7 months. Animals were kept in standard conditions and on a standard diet of the Dnipropetrovsk Medical University vivarium, all studies were conducted in accordance with modern international requirements and standards of humane treatment of animals (Council of Europe Convention of 18.03.1986 (Strasbourg); Helsinki Declaration of 1975, revised and supplemented in 2000, Law of Ukraine of February 21, 2006 №3447-IV), as evidenced by the extract from the minutes of the Biomedical Ethics Commission of Dnipropetrovsk Medical University meeting No. 3 of November 2, 2021.

The selected rats were randomly divided into three groups: group I – for the study of emotional status in the Barnes maze; group II – for the study of emotional status in the Open Field test; and group III – for the study of glutathione reductase (GR) activity in erythrocytes hemolysate. Three groups were formed in each of these groups: Experimental group (I – n = 6; II – n = 6; III – n = 28), animals of which were subjected to inhalation anesthesia with halothane (Halothan Hoechst AG, Germany), fixed in a horizontal position on a metal stand. Before the testing, all animals underwent 5-days training, i.e., during this period, each rat was placed in the center of the arena in turn every day and the time of freezing was recorded [10].

In the Open Field test, for which a standard setup was used – a large rectangular chamber (100 × 100 cm) with walls 40 cm high – the number of defecation acts was counted during 3 minutes of observation on 1st, 3rd, 7th, 14th, 21st, and 28th days of the posttraumatic period, which also allows to assess the rat’s emotional status [11].

The activity of glutathione-disulfide reductase (GR) (EC 1.8.1.7) was determined in a solution of rat’s erythrocyte’s hemolysate of the Experimental, Control and Intact groups on 1st, 3rd, 7th, 14th days of the posttraumatic period, since our previous studies have shown normalization of the activity of the antioxidant system on 14th day [10].

The erythrocyte’s hemolysate was obtained by the freeze-thaw method, for which the blood samples were first centrifuged at 2500 g for 10 minutes, the supernatant was collected and washed three times with saline. Then the erythrocytes were hemolysed by the freeze-thaw method with the addition of distilled water in a ratio of 1:5 by volume. Cell membranes were removed by centrifugation at 14,000 g for 30 minutes. The erythrocyte’s hemolysate was stored at -20 °C [12].

Glutathione reductase reduces oxidized glutathione (GSSG) in the presence of NADPH to reduced glutathione (GSH), which, upon interaction with DTNB (Elman’s reagent), forms colored reaction products that are determined spectrophotometrically with an absorbance maximum at 412 nm. Activity of GR was determined in erythrocyte’s hemolysate with tenfold diluted 0.9 % NaCl solution [13]. A mixture of 100 μl of diluted rat blood hemolysate and 0.2 ml of 4 mM oxidized glutathione solution (GSSG) was incubated in a thermostat for 5 minutes at +37 °C. Subsequently, the resulting mixture was distributed in 100 μl into Experimental, Sham and Intact microtubes. To the test sample 10 μL of 5.35 mM NADPH solution was immediately added, after which all samples were transferred to a thermostat and incubated for 10 minutes at +37 °C. The addition of 100 μL of 10 % trichloroacetic acid stopped the reaction, after which 10 μL of 5.35 mM NADPH2 solution was added to the Sham and Intact sample. Samples were incubated for another 10 minutes, then centrifuged for 15 minutes at 300 g, 200 μL of supernatant was taken into microplate wells, and 20 μL of 10 mM Elman’s reagent was added to each well. After measuring the optical density, the GR activity (μmol NADPH/min/g Hb) was determined by the difference between the absorbance at 405 nm in Experimental, Sham and Intact samples, taking into account the hemoglobin concentration in rat blood samples.

Statistical processing of the results was conducted using the software Statistica 6.1 (StatSoftinc., serial number AGAR909E415822FA). The hypothesis of normality of distribution among the studied quantitative traits was tested by the Shapiro–Wilk test. Mathematical processing included calculations of arithmetic means (M) and standard deviations (± SD). To determine the degree and nature of the relationship between the study parameters, a comparative analysis (Mann–Whitney U test) was used at the reliability thresholds of p < 0.01, p < 0.05, and the Spearman’s corre-
Results

As a result of 5-days training prior to modeling bTBI, all rats developed the same adaptive reaction in response to entering the open arena of the Barnes maze, as evidenced by a gradual decrease in freezing time (Fig. 1).

On 1st day of the experiment, rats with bTBI showed a tendency to decrease the freezing time compared to animals of the Sham (by 22 %) and Intact (by 10 %) groups. In the Sham group, the time was 13 % longer compared to the Intact animals, indicating an inhibitory effect of halothane. However, the rats of the Experimental group did not show this effect.

Comparison of the indicators on the 1st day of the experimental study with the indicators on 5th day of training in the middle of each group showed a 14 % (p < 0.01) increase in the time in the Experimental group, while in Sham and Intact rats there was a tendency to reduce the time.

On 3rd day, the period of freezing in Experimental rats was 55 % (p < 0.01) longer than in Sham and 52 % (p < 0.01) longer than in Intact animals. And inside of the Experimental group, the time prolongation was 66 % (p < 0.01) compared to 1st day after simulation of bTBI.

Subsequently, on 7th day, the freezing time in rats with bTBI was prolonged by 59 % (p < 0.01) compared with the Sham and by 51 % (p < 0.01) compared with the Intact group, while inside of the group there was a tendency (7 %, p > 0.05) to reduce the freezing time compared with the results of 3rd day. However, already on 14th day inside of the group, the reduction in the freezing time was significant compared to 7th day (34 %, p < 0.05). And in comparison with the Sham, the time increased by 45 % (p < 0.01) and by 39 % (p < 0.05) with the Intact group. On 21st and 28th days, the freezing time in the Barnes maze continued to increase in rats with bTBI by 33 % (p < 0.05) and 69 % (p < 0.01), respectively, compared with Sham rats, and by 31 % (p < 0.01) and 65 % (p < 0.01), correspondingly, compared with Intact rats. But, inside of the Experimental group, on 21st day, the time was shorter by 6.5 % (p > 0.05) compared to 14th day. And on the 28th day, the time was 47.5 % longer (p < 0.01) compared to the 21st day. The comparison of the indicators between 1st and 28th days showed a general increase in the freezing duration by 69 % (p < 0.01).

The results of defections acts number in Open Field showed no effect of halothane in rats with bTBI, while in the Sham group adaptation to the Open Field occurred on 7th day (Fig. 2).

Comparing the number of defection acts in rats of the Experimental group with those of the Sham and Intact groups (Fig. 2) were increased in Experimental group by 74 % (p < 0.05) and by 79 % (p < 0.01) appropriately. On 14th day in the Experimental group compared to 1st day defection acts number were decreased by a 79 % (p < 0.01). On 21st and 28th days, the defection acts number in Experimental animals slightly increased in the group itself, but still remained lower by 52 % (p < 0.05) and 35 % (p < 0.05) compared to other groups (Fig. 2).

As can be seen from the diagram (Fig. 3), the activity of GR in rats with bTBI is significantly higher compared to the Sham rats on 1st (72 %, p < 0.01), 3rd (61 %, p < 0.01) and 7th days (47 %, p < 0.01) and to the Intact rats on 1st (74 %, p < 0.01), 3rd (63 %, p < 0.01) and 7th days (49 %, p < 0.05).

This indicates the presence of OS in the first week of the posttraumatic period. Comparison of the indicators of GR activity in rats of the Experimental group on 1st and 14th days showed a gradient decrease by 71 % (p < 0.01). Hence, no changes in GR activity were observed (Fig. 3), while indicators characterizing the emotional state changed up to 28th day of examination (Fig. 1, Fig. 2).

To establish the presence of OS influence on the emotional status of rats with bTBI, we performed a correlation analysis (Table 1).

Discussion

Emotional disturbance is one of the most common consequences of TBI. Different types of emotional disturbance are clinically identified, including depression, apathy, ag-
In the present study, using our own device for simulation of blast trauma, we investigated the state of the emotional component of rats with mild bTBI behavior using well-known behavioral tests: the Barnes maze and the Open Field.

The Barnes maze is a large, open arena for a rat, and the animal reacts by freezing when it got on it. Consequently, by changing the freezing time that a rat spends when it got on the open surface, it is possible to assess its emotional status [16, 17].

In the present study, we observed a wavy pattern of freezing time throughout the study period. At the same time, OS was observed to have a greater negative effect on the autonomic nervous system, namely its activity level in erythrocytes of blood taken during decapitation of rats, which was manifested by a violation of the rat’s emotional status. However, a greater negative effect was on the autonomic nervous system, namely its activation on 1st and 3rd days and its depression on 7th day. Allow for the data of many modern studies that prove the high sensitivity of neurons to the effects of free radicals, our results indicate that OS, although undoubtedly involved in secondary neuronal damage, is not the leading one in bTBI [24].

Taking into account the previous results of K. Łukawski et al., J. E. Dunsmoor et al., and S. K. Oh et al., similar changes testify to development of fear, which is more regulated by the CNS, had a low degree of dependence on OS and lasted more than 7th days [24, 25, 26].

Our conclusions are in line with previous studies showing that changes similar to those in rats with bTBI indicate the development of anxiety with a transformation to a depression-like state [20, 21].

It is known that the antioxidant system is activated to protect against the effects of free radicals formed during various physiological and pathological processes and is represented by many enzymes. The main ones are superoxide dismutase, catalase, glutathione peroxidase, and glutathione. Auxiliary enzymes include glutathione reductase, glutathione S-transferase, and glucose-6-phosphate dehydrogenase, as well as metal-binding proteins: transferrin, ceruloplasmin and albumin, vitamins: alpha-tocopherol, ascorbate and beta-carotene, flavonoids and urates [27]. Glutathione reductase is an enzyme that reduces glutathione disulfide to glutathione with the participation of NADPH. This enzyme contains in the cytoplasm of cells in the form of a tripeptide — glycine-cysteine-glutamic acid. Cysteine SH-groups are extremely sensitive to the peroxides action [28, 29]. Consequently, it is clear that an increase in GR indicates the active involvement of glutathione in reducing cellular damage by free radicals in various pathologies, including TBI [25].

In view of this, the presence of OS in rats with mild bTBI was determined by the activity of erythrocyte GR compared with the level of this enzyme in Sham and Intact rats [30].

The development of negative emotionality which does not contradict the data of other scientists [19].

Also, one of the important indicators reflecting the rat’s emotional status in the experiment is the number of defection acts in the Open Field test [20, 21], changes in which we observed in all rats during the study period. In the Intact group, the changes indicated adaptation to the new environment. Comparing the number of defection acts in rats of the Experimental group with those of the Sham and Intact groups were wavylike and indicated increased activation of the autonomic system on 1st and 3rd days. Afterwards, we observed a suppression of emotionality.

Thus, our results indicate persistent impairment of emotional status in rats with mild bTBI, which coincides with the results of modern experimental studies and clinical observations [22, 23]. In addition, reliable signs of OS occurring in response to primary damage to the BW were identified. Therefore, it is worth noting that OS affects the entire body. This is evidenced by an increase in the GR activity level in erythrocytes of blood taken during decapitation of rats. At the same time, OS has an effect on certain mechanisms of nervous regulation in the acute (first week) posttraumatic period, which was manifested by a violation of the rat’s emotional status. However, a greater negative effect was on the autonomic nervous system, namely its activation on 1st and 3rd days and its depression on 7th day. Allow for the data of many modern studies that prove the high sensitivity of neurons to the effects of free radicals, our results indicate that OS, although undoubtedly involved in secondary neuronal damage, is not the leading one in bTBI [24].

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**Table 1. Correlation coefficients**

<table>
<thead>
<tr>
<th>Indicator of emotional status</th>
<th>Glutathione reductase, μmol NADPH/min/g Hb</th>
<th>1 day</th>
<th>3 day</th>
<th>7 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing time, s</td>
<td>r = -0.2</td>
<td>r = -0.2</td>
<td>r = 0.1</td>
<td></td>
</tr>
<tr>
<td>Number of defection acts</td>
<td>r = 0.7</td>
<td>r = -0.2</td>
<td>r = -0.7</td>
<td></td>
</tr>
</tbody>
</table>
The results of the correlation analysis indicate the presence of weak relationships between the freezing time in the Barnes maze and the indicators of GR. At the same time, a positive relationship of a strong degree was found between the number of defecation acts and GR on 1st day, i.e., OS contributed to the development of anxiety in rats with bTBI in the Open Field, and a negative relationship of a strong degree was also found on 7th day, i.e., OS contributed to the inhibition of the nervous system.

Thus, our study revealed reliable signs of emotional disturbance and the presence of OS in rats with bTBI. We suppose that the development of OS is one of the links in the pathogenesis of emotional disturbance, but it is not the main mechanism of brain damage. A sign of this was that changes in emotional status lasted longer than the increased activity of the GR. The correlation analysis showed a significant relationship with changes in the defecation acts number, but not with the time of freezing, which indicates an excitation of the autonomic system.

Conclusions
1. The time of freezing in the Barnes maze in Experimental rats increased by 69% (p < 0.01) on 28th day compared to 1st day, indicating the development of fear and negative emotionality after exposure to the blast wave.
2. Changes in the defecation acts number indicate the development of anxiety in the acute period (1–3 days), followed by a depression-like state, as evidenced by a decrease in defecation acts number on 14th day.
3. The activity of glutathione reductase in Experimental rats was significantly higher when compared with Sham and Intact rats, but decreased gradually inside of the Experimental group and at 14th day was almost equal to Sham and Intact values.
4. We suppose that oxidative stress is a link, but not the leading one, in the pathogenesis of emotional disturbance in rats with blast-induced traumatic brain injury.

Prospects for further scientific research are to assess the impact of oxidative stress on memory and to expand of the mild blast-induced trauma pathogenesis understanding.

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