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Altered Levels of Zinc and N-methyl-D-aspartic Acid Receptor Underlying Multiple Organ Dysfunctions After Severe Trauma

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Severe trauma can cause secondary multiple organ dysfunction syndrome (MODS) and death. Oxidative stress and/or excitatory neurotoxicity are considered as the final common pathway in nerve cell injuries. Zinc is the cofactor of the redox enzyme, and the effect of the excitatory neurotoxicity is related to N-methyl-D-aspartic acid receptor (NMDAR).





Material/Methods: We investigated the levels of zinc and brainstem NMDAR in a rabbit model of severe trauma. Zinc and serum biochemical profiles were determined. Immunohistochemistry was used to detect brainstem N-methyl-D-aspartic acid receptor 1 (NR1), N-methyl-D-aspartic acid receptor 2A (NR2A), and N-methyl-D-aspartic acid receptor 2B (NR2B) expression.

Results: Brain and brainstem Zn levels increased at 12 h, but serum Zn decreased dramatically after the trauma. NR1 in the brainstem dorsal regions increased at 6 h after injury and then decreased. NR2A in the dorsal regions decreased to a plateau at 12 h after trauma. The levels of NR2B were lowest in the death group in the brainstem. Serum zinc was positively correlated with NR2A and 2B and negatively correlated with zinc in the brain. Correlations were also found between the brainstem NR2A and that of the dorsal brainstem, as well as between brainstem NR2A and changes in NR2B. There was a negative correlation between zinc and NR2A.

Conclusions: Severe trauma led to an acute reduction of zinc enhancing oxidative stress and the changes of NMDAR causing the neurotoxicity of the nerve cells. This may be a mechanism for the occurrence of MODS or death after trauma.

MeSH Keywords: **Multiple Organ Failure • Receptors, N-Methyl-D-Aspartate • Trauma Severity Indices • Zinc**

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Background

Severe trauma causes local tissue destruction, as well as systemic inflammatory response syndrome, multiple organ dysfunction syndrome (MODS), shock, or even death [1,2]. MODS is a progressive dysfunction of 1 or more organ systems that results from an exaggerated and prolonged inflammatory response to severe illness and/or injury and is one of the most serious complications after severe trauma. The mechanism secondary to MODS or even death caused by trauma is not entirely clear. In clinical and forensic practice, we found that there are still some deaths, although there are many common factors after active treatment; further study found that this is associated with acute zinc deficiency after trauma [3–9]. We found that the levels of trace elements and antioxidant-related enzymes were changed, representing an important mechanism in the pathogenesis of multiple organ failure (MOF) or even death [10,11]. Antioxidant enzymes are a kind of antioxidant system. Oxidative stress is an imbalance between the oxidation system and antioxidation system *in vivo*, which tends to be caused by oxidation. Oxidative stress theory is important in the study of the mechanism of neurotoxicity; it has become a very popular research topic in recent years because it can explain the nerve damage and the accumulation of delayed effects [12–14]. At present, oxidative stress and/or excitotoxicity is thought to be the final common pathway of neurotoxic injury [12,15]. Excitotoxicity refers to the synaptic excitatory amino acids and its analogues, which are over-stimulated, mainly by glutamate stimulating excessive NMDAR, and cause further nerve cell degeneration and necrosis. NMDAR is a specific receptor of excitatory amino acids, and is also the main nerve pathway of the excitatory toxicity. The diversity and spatial distribution of NMDAR is an important basis of neural networks in the brain. It plays an important role in a variety of acute and chronic brain injury, particularly in the brainstem, because it contains many important life-central nuclei [16].

Therefore, we performed the present study to investigate the changes in zinc and brainstem NMDAR after application of a secondary MODS traumatic model, and to understand the possible mechanism of death due to MODS after trauma.

Material and Methods

Ethics statement

This study was conducted in strict accordance with the recommendations in the Guide for Laboratory Animal Management Regulations promulgated by the State Council, China. The Animal Care and Use Committee of the Medical College of Shantou University approved animal procedures (Number: SUMC2012XM-0039). All surgeries were performed under

sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Animals

A total of 40 healthy male New Zealand rabbits weighing 2.0–2.5 kg were used. The rabbits were quarantined for 2 weeks to allow acclimatization to the conditions prior to the experiments. They were housed individually at relative humidity of 55% with $25\pm 0.5^\circ\text{C}$ and a 12-h light/dark cycle with water and food available *ad libitum*. Each rabbit was fed 40 g of concentrated feed per day, with 40 g of zinc per Kg feed.

Experimental design

The rabbits were randomly divided into experimental and control (each, $n=8$) groups. Multiple injuries were inflicted on the pubic joints and limbs of the rabbits in the experimental groups after general anesthesia, including closed comminuted fractures of the left humerus in the left elbow joint above 1.5 cm (AIS 752804.3), complete tearing of the right knee joint posterior cruciate ligament, dislocation of the knee, abnormal AIS 804406.3 activity, and closed comminuted fractures of the lower right femur in the right knee above 1.5 cm (AIS 8518143.3). The wounded limbs were wrapped and externally stabilized with splints. Animals were fed conventional rabbit chow and were provided tap water. After injury modeling, on the first day the rabbits only drank water and ate no food, and on the second day the rabbits ate half of the normal food ration. On the third day, the rabbits ate the normal amount of food.

The human injury severity score (ISS) system is calculated as the sum of the squared scores of the most severely injured body regions [17]. According to the abbreviated injury scale of ISS [18], the score of the rabbit model was 27. The experimental group was divided into normal, injured, and death groups. Among these groups, the injured group was divided into 6-h, 12-h, and 3-day groups according to the time of death after injury. The injured rabbits that died within 24 h were classified as the death group, with the final cause of death for the rabbits in this group being a natural death. There were 8 rabbits in each experimental group.

The method of death was acute hemorrhage of the rabbit carotid artery. The rabbits were deeply anesthetized (sodium pentobarbital intraperitoneally, 100 mg/kg), and all efforts were made to minimize their suffering. Blood samples were collected simultaneously, and the brain tissue was cut in the sagittal plane, quickly frozen, and fixed in 4% paraformaldehyde.

In each rabbit, we obtained whole blood from an ear marginal vein at the following selected time points: before injury (control) and at 6 h, 12 h, and 3 days after injury. After collection,

Table 1. AST, ALT, ALB, GLB, Cr and BUN in each groups.

	AST (U/L)	ALT (U/L)	ALB (g/L)	GLB (g/L)	Cr (μmol/L)	BUN (mmol/L)
Normal group	21.27±9.10	23.13±10.84	37.98±2.38	34.27±8.82	47.63±9.74	3.69±0.94
Trauma on 6 h	91.75±13.96*	26.88±4.82	30.88±6.15	19.00±3.07*	94.50±12.86*	8.20±1.15*
Trauma on 12 h	93.87±8.90*	46.88±12.32*	25.63±4.17*	12.66±4.85*	131.62±17.20*	7.27±1.42*
Trauma on 3 d	83.87±11.33*	35.63±7.13*	29.88±6.73	21.63±11.63*	134.62±44.52*	7.81±1.50*
Dead group	82.75±9.90*	60.50±7.78*	22.88±6.62*	19.00±2.51*	111.62±16.75*	8.45±0.50*

* P<0.05 (significance compared with normal group).

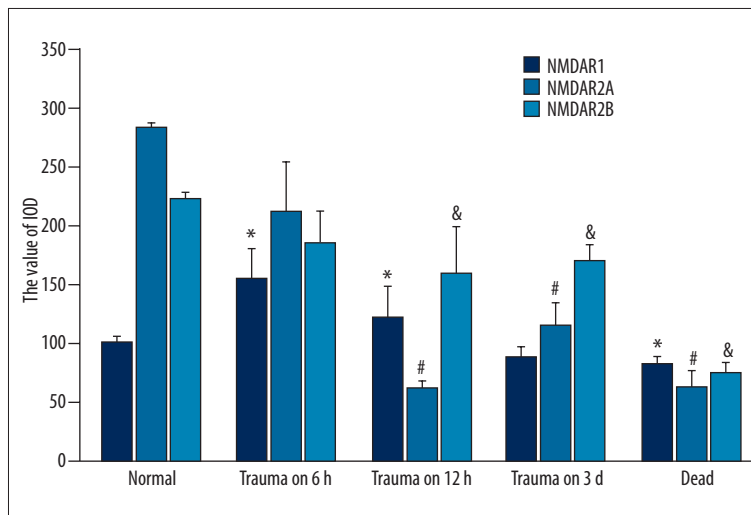


Figure 1. Alterations in the brainstem NMDAR1, NMDAR2A, and NMDAR2B expression in each experimental group. The data are shown as the mean ±SD; n=8 in each group; significance was evaluated compared with the normal group (*, #, &, p<0.05).

the serum was separated within 1 h and was stored at -30°C prior to use.

Eight rabbits were killed by carotid bloodletting at 6 h, 12 h, and 3 days post-trauma, and their brain and brainstem tissues were harvested immediately, packaged in aluminum foil, and stored at -80°C until analysis. The liver, lung, spleen, and kidney were obtained, fixed in formalin, and embedded in paraffin; 5-μm sections were cut and then stained with hematoxylin and eosin for histological examination.

Determination of Zn levels

Flame atomic absorption spectrophotometry was used to determine the serum, brain, and brainstem Zn concentrations (AA-6 800, Shimadzu Corporation).

Serum biochemical analyses

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), globulin (GLB), blood urea nitrogen (BUN), and creatinine (Cr) levels were determined using a Hitachi Automatic Analyzer 7170 (Hitachi, Japan).

Immunohistochemical analyses

Immunohistochemistry was used to detect the brainstem NR1, NR2A, and NR2B expression levels. Image-Pro Plus 6.0 was used to determine the levels of the receptor.

Statistical analysis

SPSS version 16.0 software was used to analyze the data (SPSS, Inc., Chicago, IL, USA). All the values are shown as the mean ±SD. The ANOVA and Student-Newman-Keuls tests were used for statistical analyses.

Results

Rabbit of serum biochemical profile

There were significant alterations in the experimental groups in biochemical profiles after trauma. Compared to the control group, the serum AST, ALT, ALB, GLB, BUN, and Cr levels of the experimental groups were increased significantly after the trauma, and reached their peak in the death group (p<0.05; Table 1).

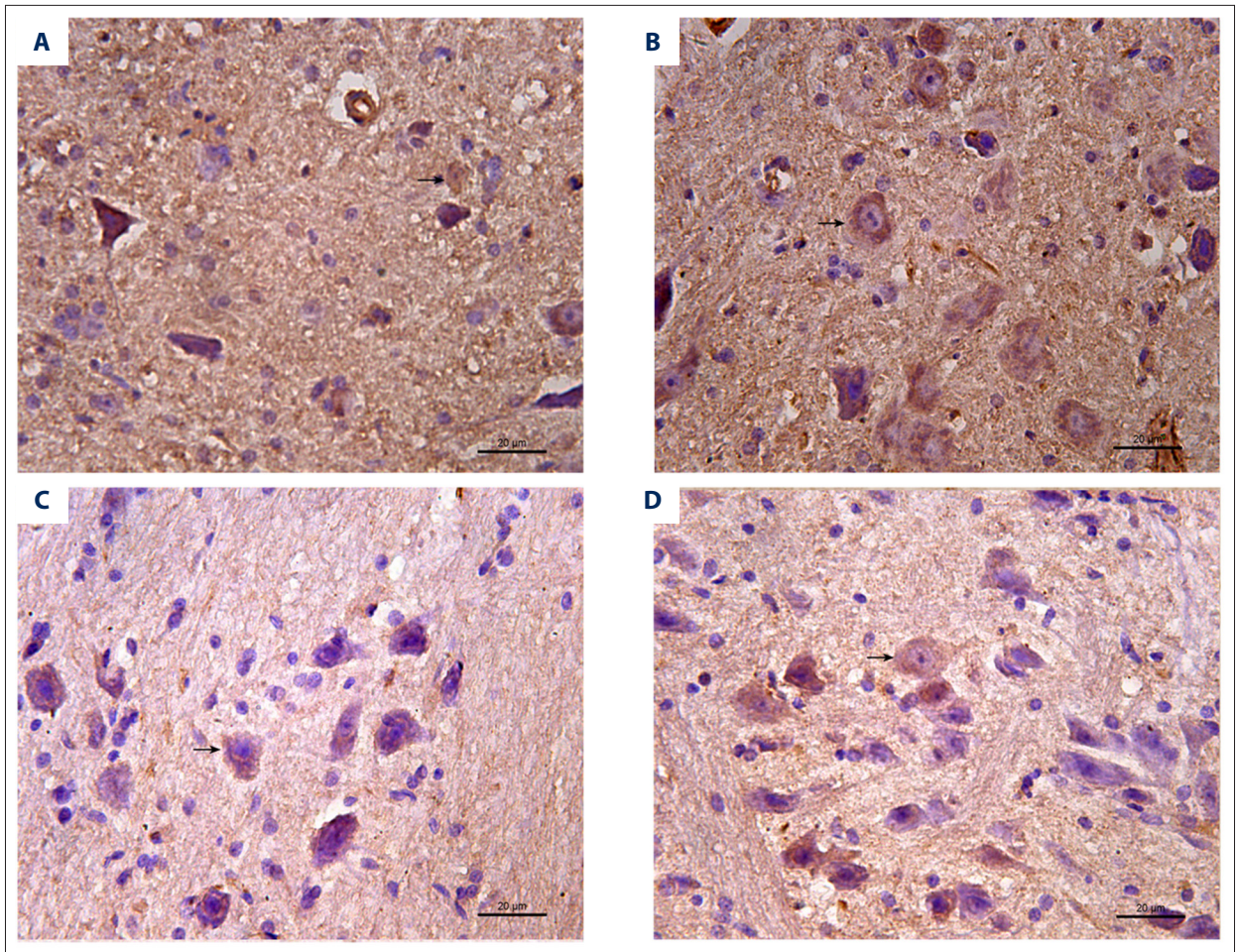


Figure 2. Immunohistochemical expression of NMDAR1 in the dorsal brainstem. (A–D) represent the trauma at 6 h, 12 h, 3 days, and in the dead group, respectively.

Immunohistochemical detection of brainstem NR1, NR2A, and NR2B

In the dorsal brainstem 6 h after injury, NR1 levels were higher than in the control group, but at 12 h, NR1 levels were significantly decreased compared with those at 6 h after injury and approached baseline levels by 3 days. The death group had lower NR1 levels than those of the normal group ($p < 0.05$).

In the dorsal brainstem, the injured and death groups displayed lower NR2A levels than those of the normal group, with the lowest levels found in the death group ($p < 0.05$). The NR2B levels were lower in the injured group than in the control group; this difference was not significant among the injured groups, but the injured groups displayed significantly higher levels than in the death group (Figures 1–4).

Serum, brain, and brainstem Zn profiles

Compared with pre-injury, serum zinc levels decreased by 6 h after injury and decreased further 12 h after injury; however, serum zinc levels increased 3 days after injury. The content of zinc in the brain and brainstem increased 6 h after trauma and continued to increase over the course of 3 days after the injury compared to the normal group (Figure 5).

Correlation between zinc and brainstem NR1, NR2A, and NR2B levels

There was a positive correlation between changes in the blood zinc levels and dorsal brainstem NR2A ($r = 0.629$, $P < 0.05$) and NR2B ($r = 0.696$, $P < 0.05$). There was a negative correlation between brain zinc levels and dorsal brainstem NR2A expression ($r = -0.702$, $P < 0.05$). There was a negative correlation between brain zinc levels and NR2B expression in the dorsal brainstem ($r = -0.568$, $P < 0.05$). There was a negative correlation between

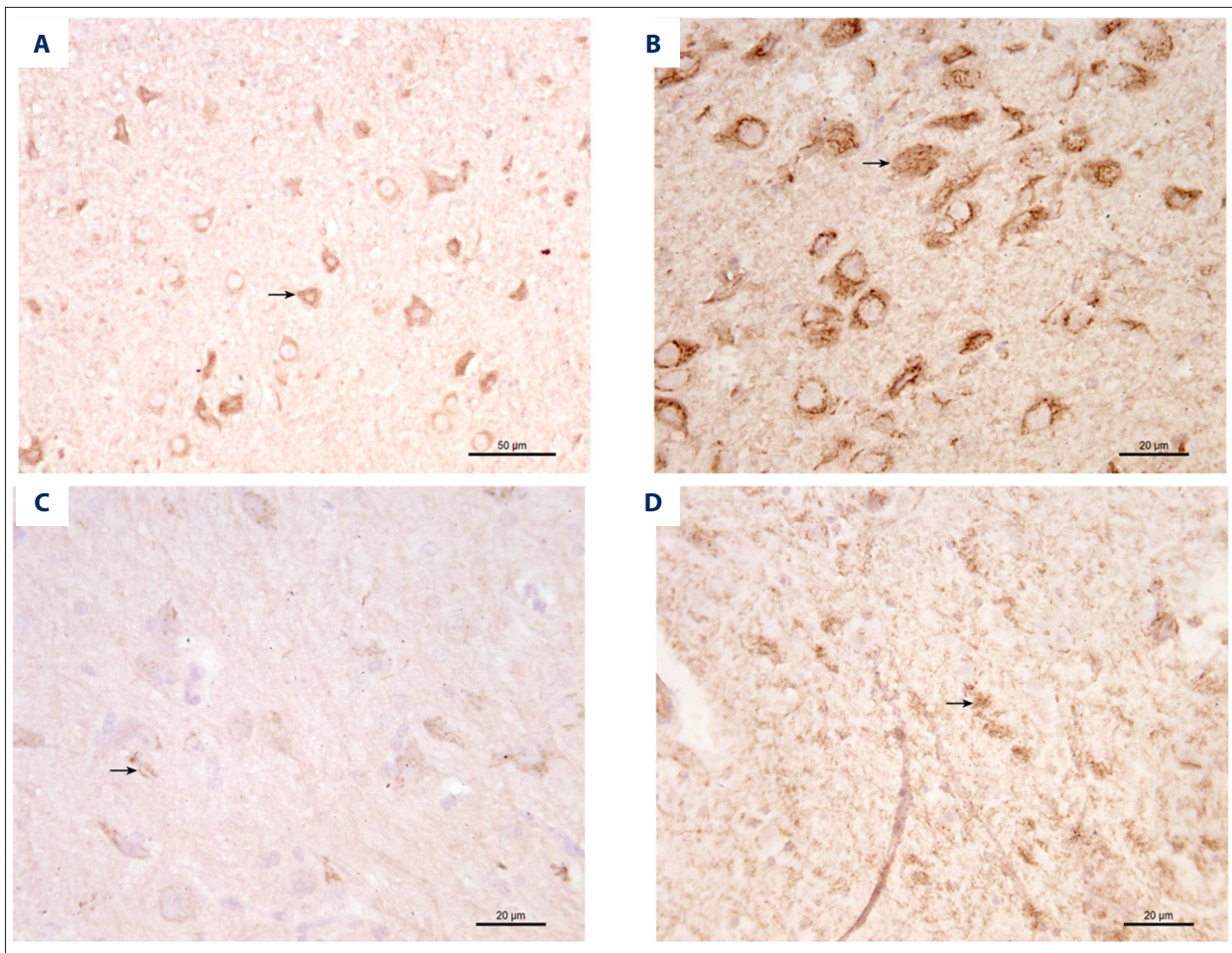


Figure 3. Immunohistochemical expression of NMDAR2A in the dorsal brainstem. (A–D) represent the trauma at 6 h, 12 h, 3 days, and in the dead group, respectively.

the brain stem zinc levels and dorsal brainstem NR2A expression ($r=-0.616$, $P<0.05$).

Discussion

Changes and significance of zinc were closely associated with oxidative stress after trauma

Severe trauma can lead to a variety of inflammatory mediators and inflammatory cytokines release in the body, causing SIRS and oxidative stress. Oxidative stress is an oxygen free radical (ROS)-generated cytotoxic effect. ROS is a by-product in the normal and abnormal metabolic processes using the molecular oxygen produced. Oxidative stress theory has an important position in the study of the mechanism of neurotoxicity. It is very popular in recent years because it can explain the nerve damage and the accumulation of delayed effects [12–14]. Under normal circumstances, the generation and elimination of ROS in nerve tissues is in a state of dynamic equilibrium [14]. The

structure and function of the nervous system is destroyed by a variety of adverse factors; the dynamic balance is destroyed and cell function and integrity is damaged.

In this experiment, serum zinc decreased sharply after injury and continued to decrease 12 h after injury. Zinc levels remained low at 3 days after injury and in the death group, suggesting that severe trauma can lead to an acute drop in serum zinc [17]. Zinc content in the brain and brainstem tissue gradually increased after severe trauma, and was significantly higher in the death group but lower in the survival group after injury. Because zinc plays an important role in the antioxidant system, brain tissue can continue to redistribute zinc to a certain degree after severe trauma to improve antioxidant defense [19–21].

Changes in and significance of NMDAR are closely related to excitotoxicity after severe trauma

In nerve cell damage, in addition to oxidative stress, a very important factor is excitatory neurotoxicity. Excitotoxicity is caused

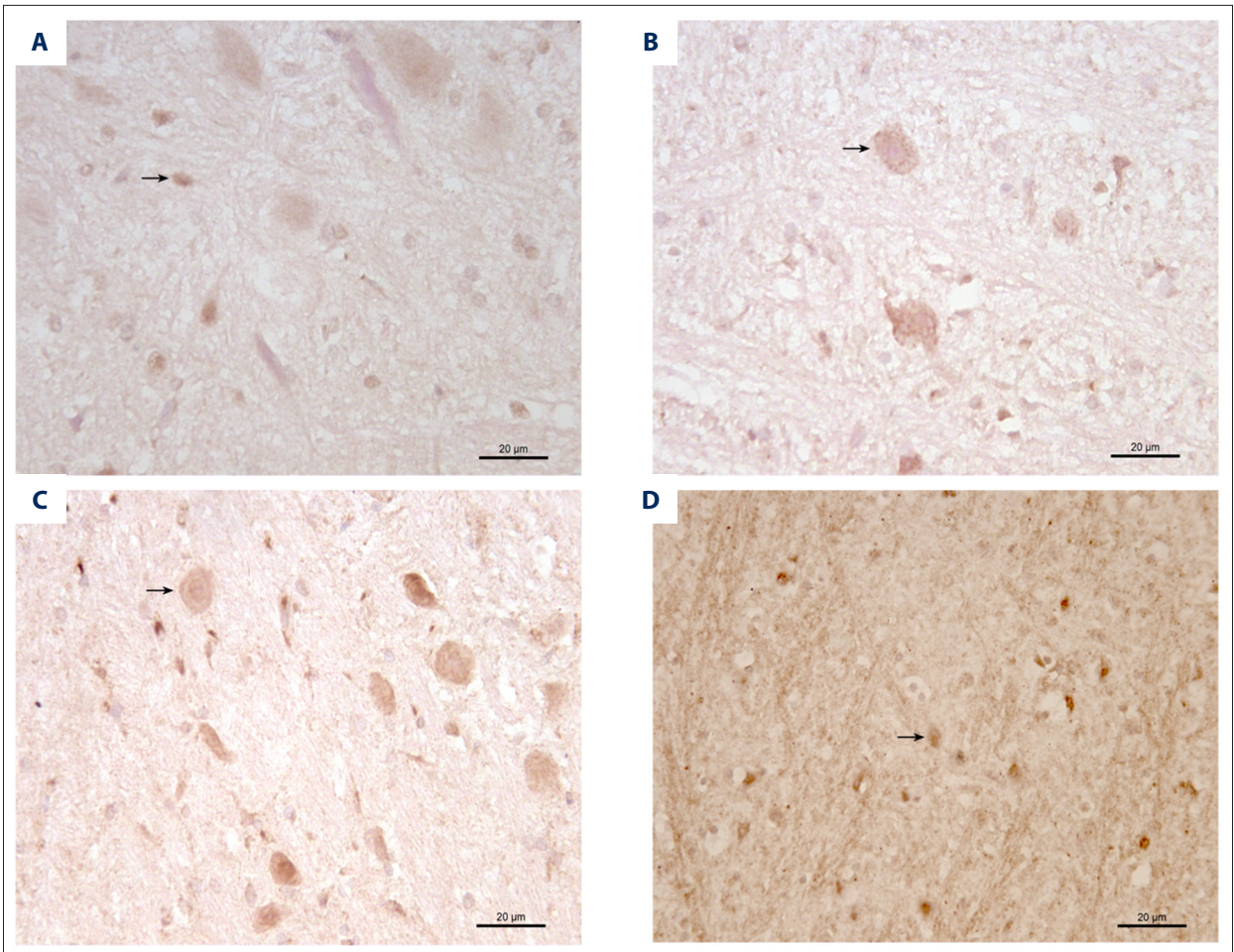


Figure 4. Immunohistochemical expression of NMDAR2B in the dorsal brainstem. (A–D) represent the trauma at 6 h, 12 h, 3 days, and in the dead group, respectively.

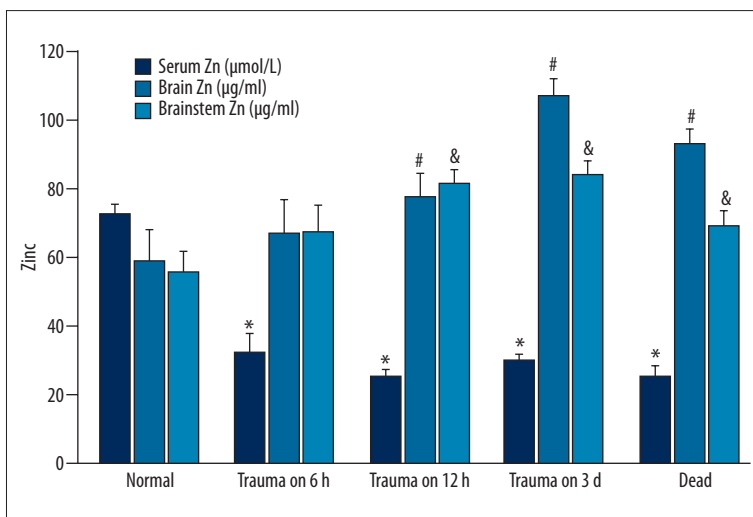


Figure 5. Alterations in the serum, brain, and brainstem Zn concentrations after major trauma. The data are shown as the mean \pm SD; n=8 in each group; significance was evaluated compared with the normal group (*, #, &, p<0.05).

by a variety of oxygen free radical and inflammatory medium-stimulated glutamate. Among them, NMDAR is the main receptor, causing nerve cell degeneration and necrosis. The activity of NMDAR is associated with brain development. Their excessive activity can cause brain damage. Clinically, NMDA receptors are closely related to cerebral ischemia, hypoxia, hypoglycemia, epilepsy, and traumatic brain injury.

NR1 is an important member of the ionotropic glutamate receptor family, whose role is mediated by glutamate; NR1 plays an important role under physiological conditions [22]. This experiment confirmed that NR1 content in the dorsal brainstem was higher at 6 h following injury and returned to near normal values after 3 days. These results show that the body manipulates NMDAR subunits by compensatory mechanisms after injury, resulting in the enhanced neurotoxicity of excitatory amino acids during this time [23]. The NR1 subunit itself has all the features of NMDAR. The results are consistent with the finding that the ischemia and hypoxia in brain ultimately results in neurons damage by the activation of NMDAR.

In this study, we found that N-methyl-D-aspartic acid receptor 2 (NR2) showed significant changes after injury. NR2A and 2B are 2 distinct regulatory protein subunits. Presumably, the regulatory subunits decrease after trauma, reducing inhibition of the binding site for receptor activity. Inhibitor binding may be reduced, leading to a reduction in the final inhibition. Related research has confirmed that there is a difference between the expression of NR2A and 2B in the brain of rats, which provides

the molecular basis for the expression of NMDAR at different times and in various locations in brain tissue [24]. We showed that NR2A and 2B displayed a higher degree of sensitivity to changes in serum zinc levels, suggesting that, as regulatory protein components, they are more sensitive to the environment, particularly the outer periphery of post-traumatic stress with strong stimulation [25,26].

The results also confirmed that levels of zinc in the blood and NR1 in the brainstem are not correlated, most likely due to increased blood zinc loss, intake, and redistribution. In addition, NR1 expression is a large-content functional unit that is subject to greater effects, particularly during the compensatory stage. Based on the correlation of brain and brainstem zinc with NR2A and 2B expression, we hypothesize that after severe trauma NR2 does not require as much inhibition to achieve the suppression effect. The acute increase in brain zinc content met this requirement. The correlation between zinc and the brainstem NR2A and 2B subunits indicated that zinc had a better affinity in the brain for inhibiting NR2A. This finding is consistent with the results of similar studies [27–29].

Conclusions

After severe trauma, acute lack of zinc reduces the antioxidant activity, and changes in NMDAR lead to enhanced excitatory neurotoxicity, causing substantial damage to nuclei of the brainstem.

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