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Effects of Transcranial Direct Current Stimulation on Motor and Cognitive Dysfunction in an Experimental Traumatic Brain Injury Model

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ABSTRACT

AIM: To investigate the therapeutic and neuroprotective effects of transcranial direct current stimulation (tDCS) application on the traumatic brain injury (TBI)-induced glutamate and calcium excitotoxicity and loss of motor and cognitive functions.

MATERIAL and METHODS: Forty rats were equally divided in the sham, TBI, tDCS + TBI + tDCS, and TBI + tDCS groups. Mild TBI was induced by dropping a 450-g iron weight from a height of 1 m onto the skull of the rats. The tDCS + TBI + tDCS group was prophylactically administered 1 mA stimulation for 30 min for 7 days starting 5 days before inducing TBI. In the TBI + tDCS group, tDCS (1 mA for 30 min) was administered 2 h after TBI, on days 1 and 2. Cognitive and locomotor functions were assessed using the novel object recognition and open field tests. The calcium, glutamate, and N-methyl-D-aspartate receptor 1 (NMDAR1) levels in the hippocampus were measured using enzyme-linked immunosorbent assay.

RESULTS: Although the motor and cognitive functions were substantially reduced in the TBI group when compared with the sham, they improved in the treatment groups ($p<0.05$). The calcium, glutamate, and NMDAR1 levels were considerably higher in the TBI group than in the sham ($p < 0.001$). However, they were considerably lower in the tDCS + TBI + tDCS and TBI + tDCS groups than in the TBI groups ($p < 0.05$). In particular, the change in the tDCS + TBI + tDCS group was higher than that in the TBI + tDCS group.

CONCLUSION: Application of tDCS before the development of TBI improved motor and cognitive dysfunction. It demonstrated a neuroprotective and therapeutic effect by reducing the excitotoxicity via the regulation of calcium and glutamate levels.

KEYWORDS: Calcium, Glutamate, N-methyl-d-aspartate receptor, Transcranial direct current stimulation, Traumatic brain injury, Rat

ABBREVIATIONS: TBI: Traumatic brain injury, **tDCS:** Transcranial direct current stimulation, **NMDAR:** N-methyl-d-aspartate receptor, **OF:** Open field, **NOR:** Novel object recognition, **ELISA:** Enzyme-linked immunosorbent assay

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INTRODUCTION

Traumatic brain injury (TBI) is defined as damage to the brain caused by direct and indirect forces. A blow to the brain from outside the body can cause temporary or permanent neurological dysfunction. Furthermore, repetitive head trauma can result in chronic traumatic encephalopathy (15). In particular, in people who have been boxing professionally for several years, changes in mood, memory, and behavior have been observed. Recently, it has been acknowledged that repeated TBIs in professional players in various sports can lead to increasingly serious consequences (17). Most cases of TBI are encountered in martial sports, and circumstances similar to martial sports can be encountered in professional hockey, football, and war (15). Because of this circumstance, permanent damage can occur and affect people's lives. There is a need to develop treatment protocols with neuroprotective effects that can also be applied prophylactically in people at high risk of developing TBI. This could minimize the damage that individuals who are at high risk for recurrent head trauma would experience in their sports or professional life. TBI is a major cause of disability worldwide. Because the brain is the most vulnerable and complex organ of the body, TBIs affect the life of the person in several ways. This leads to physical, cognitive, and behavioral losses. Head trauma is a lethal, disabling pathology that requires long-term treatment and care, and it statistically ranks fourth among the causes of death (3,6). Although it is a common and serious health concern, making a diagnosis and predicting the prognosis remain challenging (3). Clinically, TBI may result in changes in cognitive characteristics, such as memory loss, perception difficulties, distraction, and logical thinking, as well as physical issues, such as partial or complete paralysis, balance disorders, swallowing difficulty, and speech disorders. Unless TBI is treated early, the mortality rate is quite high (3,6).

TBI increases the extracellular glutamate concentration (26). Disruption of calcium-mediated exocytosis and presynaptic membrane-bound ion pumps causes glutamate release from neurons because of depolarization (3,5). This reportedly causes a toxic increase in the intracellular calcium concentration. The association of intracellular N-methyl-D-aspartate receptors (NMDARs) with reactive oxygen and nitrogen particles causes a lethal influx of ions, particularly calcium ions, after glutamatergic stimulation (18). During excitotoxicity, mitochondria maintain intracellular calcium balance by retaining excess free calcium (3).

The effects of several pharmacological agents, such as anti-inflammatory drugs, excitotoxicity-blocking drugs, antiapoptotic agents, calcium channel blockers, free radical scavengers, steroids, and statins, have been tested in different animal models to limit biochemical damage and cell death after a TBI. In animal experiments, statins have reduced glial activation and inflammatory response, which cause cerebral ischemia and secondary neuronal damage in closed-head traumas (17). Increasing knowledge about the physiopathology of head traumas, application of appropriate treatment methods by identifying the physiology of TBI-induced secondary neuronal damage, and advanced developments in intensive patient care techniques have reduced mortality rates and significantly improved the prognosis. Analysis of the TBI pathophysiology demonstrates that excitotoxicity of the NMDARs and free radical-induced cell death processes play a major role in causing neuronal damage (18).

Although there are studies on drug treatment of TBI-induced learning and memory impairment, studies using transcranial direct current stimulation (tDCS) neuromodulation, a noninvasive method, are limited; tDCS is a relatively novel technique (1,4). tDCS induces changes in the resting membrane potential via the modulation of sodium and calcium channels. tDCS effectively acts on voltage-gated calcium channels, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), and NMDARs (21). Although there are studies on the treatment of TBI-induced learning and memory impairment using different methods, there are no studies that have demonstrated the effect of tDCS stimulation on glutamate-associated cognitive and molecular mechanisms. Thus, herein, we have aimed to evaluate the effects of tDCS on TNI-induced motor and cognitive functional changes as well as on the levels of NMDAR1, calcium, and glutamate. Furthermore, we evaluated the prophylactic activity of tDCS in people at risk of TBI before the development of TBI. Thus, both the neuroprotective and therapeutic efficacy of tDCS treatment were investigated.

MATERIAL and METHODS

Compliance with Ethical Standards

The authors declare no competing financial interests. All animal use and experimental protocols were approved and implemented by Erciyes University (24/015).

Animals and Experimental Design

A total of 40 three-month-old, male, Wistar albino rats weighing 250–300 g were divided into the sham, TBI, tDCS + TBI + tDCS, and TBI + tDCS groups (Figure 1). A mild TBI (mTBI) model was created using the Marmarou method (19). Locomotor activities and cognitive function were assessed using the open field (OF) and novel object recognition (NOR) tests, respectively. After the experiments, the rats were euthanized, the hippocampus was removed and calcium, glutamate, and NMDAR1 levels were measured using enzyme-linked immunosorbent assay (ELISA).

Marmarou Weight-Drop Model

In our study, Marmarou's weight-drop method, which is the most preferred weight-drop model, was used (19). The rats were anesthetized with 5% isoflurane. Thereafter, a moderate chronic traumatic encephalopathy model was created by dropping a weight of 450 g from a height of 1 m directly onto a steel disk (14,20).

Transcranial Direct Current Stimulation Application

The animal DCS Stimulator was used in the experiments. Anodal tDCS stimulation (1 mA for 30 min) was applied with a disk electrode that was placed on the heads of rats under

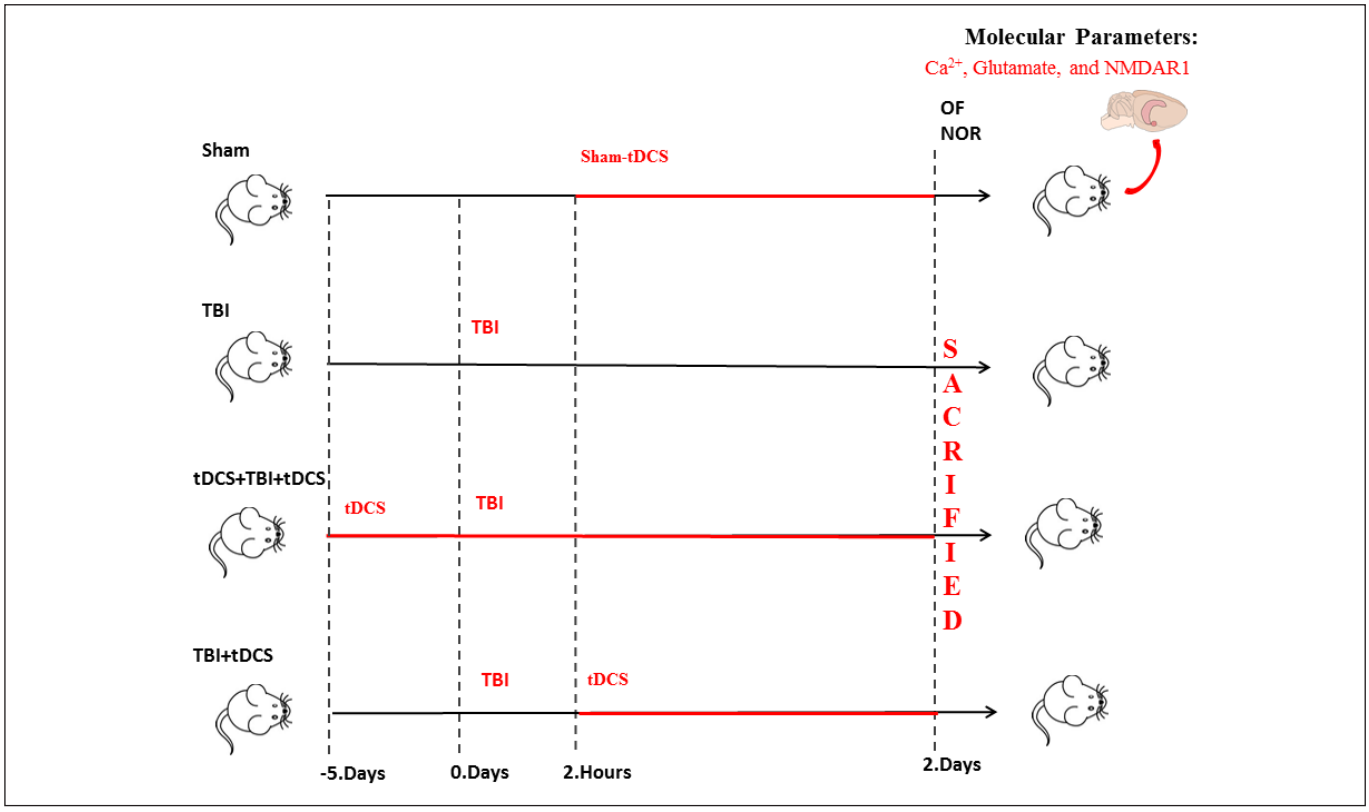

Figure 1: Experimental design.

isoflurane anesthesia. tDCS was applied in the sham, tDCS + TBI + tDCS, and TBI + tDCS groups.

Behavioral Tests

Open-Field test

Locomotor activity was assessed in a setup with a base of 80 x 80 cm. The rats were placed in the center of the area, monitored, and recorded using a video camera for 5 min. The total distance covered (cm), velocity (cm/s), and number of squares entered were noted to evaluate the locomotor activity (2).

Novel object recognition test

Short-term memory was assessed using the NOR test. The time spent with novel objects was recorded for each rat (2). The discrimination index and time spent with the novel object(s) were analyzed in the NOR test. Discrimination index = [(Time spent with the novel object – time spent with the old object)/total time) x 100.

Tissue Collection

The rats were decapitated on day 2 after the behavioral experiments. For the biochemical analyses, the motor cortex tissue was dissected from the brain and stored at –80°C. The hippocampus was homogenized in phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatants were used in the ELISA analyses.

Biochemical Analysis

Enzyme-linked immunosorbent assay

The levels of calcium, glutamate, and NMDAR1 were quantified using ELISA kits. The concentrations were calculated from the calcium, glutamate, and NMDAR1 absorbance values in the supernatants for the standard curve. The values were normalized to that of total protein and expressed as pg/mg of tissue protein.

Protein measurements

The protein concentration in the hippocampus tissues were measured using a modified Bradford assay (Pierce Chemical Company, Rockford, IL, USA) at 595 nm.

Statistical Analysis

SPSS was used for the statistical analyses of data obtained from the behavioral and biochemical experiments. The results are presented as mean ± standard error of the mean (SEM). A p-value of < 0.05 was considered statistically significant. The one-way ANOVA and Tukey's test for post hoc analysis were used to analyze data with a normal distribution in the Shapiro–Wilk test.

RESULTS

The total distance covered, velocity, and number of squares entered in the OF test were significantly lesser in the TBI group than in the sham group ($p<0.01$) (Table I, Figure 2.). The total

Table I: Locomotor Activity Results of Experimental Groups in OF

	Control		TBI		TBI+tDCS		tDCS+TBI+tDCS	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Total Distance (cm)	1450.79	91.97	**860.00***	30.83	**1120.00*****	38.37	**1250.00****	25.00
Velocity (cm/s)	5.25	0.22	**3.50***	0.10	**4.00****	0.16	**4.30****	0.08
The number of squares entered	70.00	2.74	**55.00***	1.05	**63.00****	1.28	**65.00****	1.30

TBI: *Traumatic brain injury,* ***tDCS:*** *Transcranial direct current stimulation,* ***OF:*** *Open field. *p<0.01 compared to sham, **p<0.05, ## p<0.01 compared to TBI, one-way ANOVA test, followed by Tukey post hoc test. All data are presented as means ± SEM, n=10 for each group).*

Table II: Learning Results of Experimental Groups in NOR

	Control		TBI		TBI+tDCS		tDCS+TBI+tDCS	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Discrimination index (%)	55.00	1.69	36.00*	4.60	42.00**	2.32	46.00***	3.00
Exploration time of the novel object (s)	40.67	1.18	29.00*	2.51	34.00**	1.84	36.00**	2.00

TBI: *Traumatic brain injury,* ***tDCS:*** *Transcranial direct current stimulation,* ***NOR:*** *Novel object recognition. *p<0.01 compared to sham, **p<0.05, ***p<0.01 compared to TBI, one-way ANOVA test, followed by Tukey post hoc test. All data are presented as means ± SEM, n=10 for each group).*

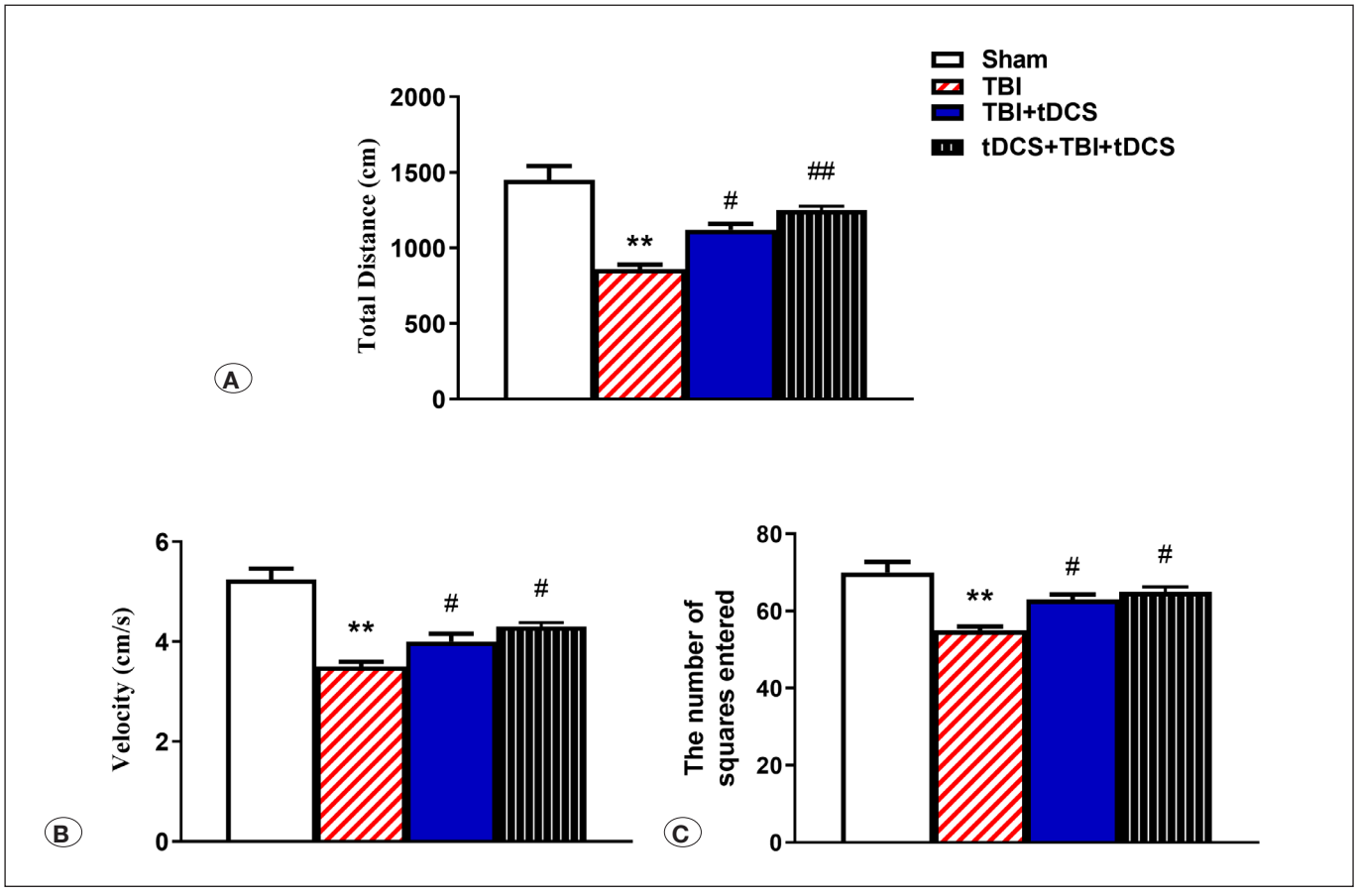

Figure 2: Behavioral results of the experimental groups. **A)** Total distance (cm), **B)** Velocity (cm/s), and **C)** Number of squares entered in the open field test. ** p<0.01 vs. sham, # p<0.05 vs. TBI, n=10 for each group.

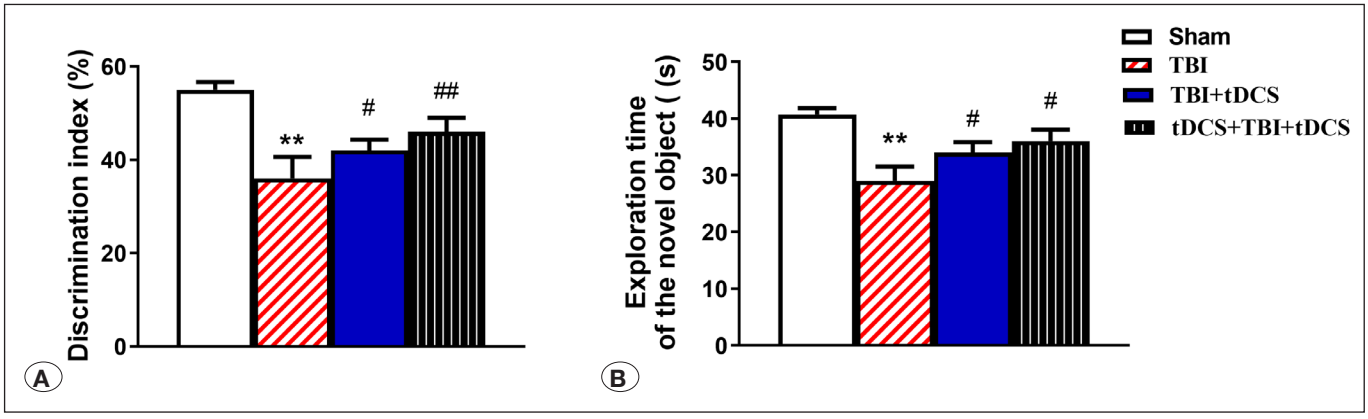

Figure 3: Learning results of the experimental groups. **A)** Discrimination index (%) and **B)** time spent exploring the novel object (sec) in the novel object recognition test. **p<0.01 vs. sham, #p<0.05 vs. TBI, n=10 for each group.

Table III: ELISA Results of Ca^{2+}, Glutamate and NMDAR1 Levels

	Control		TBI		TBI+tDCS		tDCS+TBI+tDCS	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Ca^{2+} (µg/ml/g protein)	1.10	0.05	**1.53***	0.04	**1.35****	0.03	**1.28****	0.03
Glutamate (µg/ml/g protein)	0.80	0.05	**1.20***	0.07	**0.99****	0.02	**0.92*****	0.02
NMDAR1 (µg/ml/g protein)	1.11	0.05	**1.90***	0.21	**1.75****	0.12	**1.64****	0.12

TBI: *Traumatic brain injury,* ***tDCS:*** *Transcranial direct current stimulation,* ***NMDAR:*** *N-Methyl-D-Aspartate receptor, *p<0.01 compared to sham, **p<0.05, ***p<0.01 compared to TBI, one-way ANOVA test, followed by Tukey post hoc test. All data are presented as means ± SEM, n=10 for each group).*

distance covered, velocity, and number of squares entered were significantly more in the tDCS + TBI + tDCS and TBI + tDCS groups than in the TBI group (p<0.05). The discrimination index and time spent exploring the novel object in the NOR test were significantly lesser in the TBI group than in the sham group (p<0.01) (Table II, Figure 3). Both the discrimination index and time spent exploring the novel object were significantly more in the tDCS + TBI + tDCS and TBI + tDCS groups than in the TBI group (p<0.05). The calcium, glutamate, and NMDAR1 levels in the hippocampus were significantly higher in the TBI group than in the sham group (p<0.01) (Table III). The calcium, glutamate, and NMDAR1 levels were significantly lower in the tDCS + TBI + tDCS and TBI + tDCS groups than in the TBI group (p<0.05).

DISCUSSION

TBI following head trauma remains an important health concern despite groundbreaking advances in medicine. The primary injury occurs immediately after trauma. However, primary damage alone is not responsible for the trauma-induced brain damage. Secondary injury is observed hours or days after the primary injury. Secondary injury is associated with a poor prognosis in patients with TBI (17). The mechanisms leading to secondary damage include neurotransmitter release, reactive oxygen species generation, calcium-dependent cell damage, gene activation, mitochondrial dysfunction, and inflammation. The hippocampus plays a fundamental role in neuronal plasticity, perception, memory, emotion, spatial navigation, and orientation. TBI causes learning and memory impairment. In this study, learning and memory impairment were found in the TBI group. Furthermore, tDCS stimulation produced a therapeutic effect on the impaired learning and memory as well as the locomotor activity. There is a need to develop treatment protocols that not only produces neuroprotective effects but also can be used prophylactically in individuals at high risk of developing TBI. This could minimize the damage that individuals who are at high risk for recurrent head trauma would experience in their sports or professional life. In our study, the findings in the tDCS + TBI + tDCS group demonstrated the prophylactic efficacy of tDCS. Thus, tDCS can be considered a treatment option for TBI, as well as a prophylactic against the development of TBI.

Decreased locomotor activity in animals indicates a loss of motor function. In this study, the locomotor activity in rats decreased after mTBI. However, tDCS application reduced the loss of locomotor activity. Moreover, the locomotor activity of tDCS + TBI + tDCS and TBI + tDCS groups was similar to that of the sham group. Thus, tDCS application not only improved the mTBI-induced loss of motor function but also increased the locomotor activity to almost normal. Similar to the locomotor activity results, tDCS application also decreased the TBI-induced cognitive dysfunction. Our study findings are similar to those of previous studies (8,11,27). Kim and Han

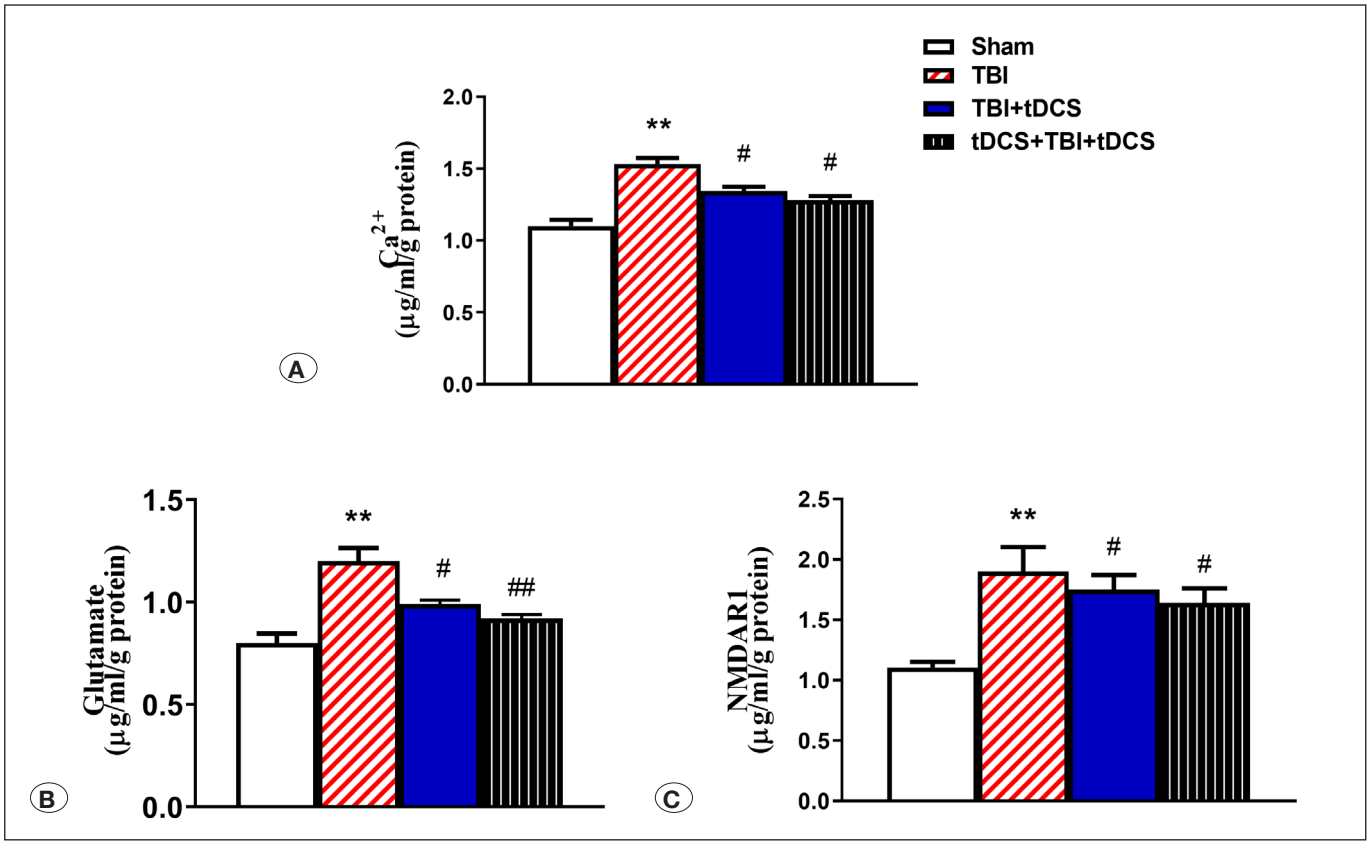

Figure 4: Enzyme-linked immunosorbent assay results of the experimental groups. **A)** Calcium, **B)** glutamate, and **C)** N-methyl-d-aspartate receptor 1 levels in the hippocampus. **p<0.01 vs. Sham, #p<0.05 vs. traumatic brain injury, n=10 for each group.

demonstrated that application of 0.2 mA of anodal tDCS for 30 min reduces the loss of motor function after TBI (11). Yu et al. also reported that application of 0.2 mA of anodal tDCS for 30 min improved the loss of motor and cognitive function caused by TBI (27). In our study, application of anodal tDCS (0.5 mA for 30 min for 2 days) reduced the loss of locomotor and cognitive function caused by mTBI.

Glutamate is the main excitatory neurotransmitter involved in intracellular communication, synaptic plasticity, cell growth, and neuronal as well as glial cell death. Glutamate stimulates calcium channel receptors such as NMDA, leading to ischemic neuronal damage and intracellular calcium increase. These trigger events which consequently result in enzymatic cellular death (16,22). Yi and Hazell demonstrated that brain trauma causes an increase in the extracellular concentration of the excitatory neurotransmitter glutamate (26). Disruption of calcium-mediated exocytosis and presynaptic membrane ion pumps causes glutamate release from neurons because of depolarization (3,5). This neurotransmission is believed to cause a toxic increase in intracellular calcium concentration. The results of our study are similar to those reported in the literature. We found a significant increase in the glutamate and calcium levels following TBI. tDCS application decreased the elevated levels of glutamate and calcium. In particular, it demonstrated a significant therapeutic effect, as well as a protective effect, in the tDCS + TBI + tDCS group. Repeated head traumas can cause chronic traumatic encephalopathy (15), which may manifest as memory disorders, altered moods, and behavioral changes, especially in professional boxers after several years of boxing. Recently, it has been recognized that repeated TBIs in professional players in various sports lead to progressively more serious consequences (15). Although most of these incidents occur in martial sports, similar situations can also be observed in professional hockey, football, and war (15). Repeated TBIs can cause permanent damage and affect people's lives.

Traumatic neural damage causes an increased conduction response to AMPAR agonists. Damaged neurons demonstrate increased AMPAR ion transmission, hyperexcitability, increased intracellular free calcium concentrations, and sensitivity to other synthetic glutamate receptor analogs at non-toxic concentrations (7). When there is a decrease in AMPAR desensitization or hypersensitivity, the neurotoxicity that develops due to a TBI-induced short-term increase in synaptic glutamate may lead to hyperexcitability, cell damage, and death. In addition to AMPAR-dependent hyperexcitability, increased NMDAR activity has also been noted in several TBI studies. The association of intracellular NMDARs with reactive oxygen and nitrogen particles causes a lethal ion influx, particularly that of calcium ions, after glutamatergic stimulation (18). During excitotoxicity, mitochondria maintain intracellular calcium balance by retaining excess free calcium (3). Stud-

ies have illustrated that cerebral ischemia causes excessive accumulation of extracellular glutamate due to increased glutamate release or impaired reuptake. This in turn leads to excessive activation of the NMDARs and subsequent cell death with excessive calcium entry into the neurons (23,25). Head traumas or ischemic strokes initiate the toxic pathways of the NMDARs, resulting in death of the neurons (9,10,13,24). In our study, a rise in NMDAR1 levels was observed after TBI. This increase may have been associated with the excessive stimulation of NMDARs by glutamate, which increases because of the head trauma-induced secondary damage. tDCS application reduced the NMDAR1 levels.

Limitations

This study had some limitations. We only used female rats, and we did not evaluate the effects of long-term application of tDCS. Furthermore, evaluating the effects of tDCS on neuroinflammation, oxidative stress, and apoptosis would have significantly contributed to the study's impact.

CONCLUSION

In this study, we demonstrated that tDCS application before and after TBI positively affected the locomotor activity and learning in rats. In particular, early treatment with tDCS reduced the loss of locomotor activity and cognitive function. tDCS demonstrated neuroprotective and therapeutic effects in relation to neural damage by reducing calcium and glutamate excitotoxicity. Anodal tDCS stimulation ameliorated the TB-induced impairment of learning, memory, and motor performance. Furthermore, tDCS stimulation contributed to the neuroprotective process by reducing the TBI-induced increase in calcium and glutamate levels. Thus, prophylactic application of tDCS demonstrates a neuroprotective effect, especially in individuals at high risk for developing TBI. Additionally, tDCS application acts as an antagonist of NMDAR, which plays a vital role in TBI. Although the results of our study indicate that tDCS stimulation plays a role in the regulation of synaptic glutamate levels, the mechanism by which glutamate receptors contribute to the process requires further investigation. In addition, the effect of tDCS application to the hippocampal CA1 and CA3 regions, which are the most vulnerable to TBI, requires further investigation.

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AUTHORSHIP CONTRIBUTION

Study conception and design: GA, DK, AY
Data collection: GA, EK, HEO
Analysis and interpretation of results: GA, DK, CC, HC, YE
Draft manuscript preparation: GA, DK, FD, TG, AY
All authors (GA, FD, TG, AY, DK, EK, HEO, CC, HC, YE) reviewed the results and approved the final version of the manuscript.

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