Using dual polarities of transcranial direct current stimulation in global cerebral ischemia and its following reperfusion period attenuates neuronal injury

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Abstract

Multiple neuronal injury pathways are activated during cerebral ischemia and reperfusion (I/R). This study was designed to decrease potential neuronal injuries by using both transcranial direct current stimulation (tDCS) polarities in cerebral ischemia and its following reperfusion period. Ninety rats were randomly divided into six groups. In the sham group, rats were intact. In the I/R group, global cerebral I/R was only induced. In the I/R + c-tDCS and I/R + a-tDCS groups, cathodal and anodal currents were applied, respectively. In the I/R + c/a-tDCS, cathodal current was used in the cerebral ischemia and anodal in the reperfusion. In the I/R + a/c-tDCS group, cathodal and anodal currents were applied in the I/R, respectively. Hippocampal tissue was used to determine the levels of IL-1β, TNF-α, NOS, SOD, MDA, and NMDAR. Hot plate and open field tests evaluated sensory and locomotor performances. The cerebral edema was also measured. Histological assessment was assessed by H/E and Nissl staining of the hippocampal CA1 region. All tDCS modes significantly decreased IL-1β and TNF-α levels, especially in the c/a-tDCS. All tDCS caused a significant decrease in MDA and NOS levels while increasing SOD activity compared to the I/R group, especially in the c/a-tDCS mode. In the c-tDCS and a/c-tDCS groups, the NMDAR level was significantly decreased. The c/a-tDCS group improved sensory and locomotor performances more than other groups receiving tDCS. Furthermore, the least neuronal death was observed in the c/a-tDCS mode. Using two different polarities of tDCS could induce more neuroprotective versus pathophysiological pathways in cerebral I/R, especially in c/a-tDCS mode.

Highlights

Multiple pathways of neuronal injury are activated in cerebral ischemia and reperfusion (I/R).
Using tDCS could modulate neuroinflammation and oxidative stress pathways in global cerebral I/R.
Using c/a-tDCS mode during cerebral I/R causes more neuroprotective effects against neuronal injuries of cerebral I/R.

Keywords Transcranial Direct Current Stimulation · Brain Ischemia–Reperfusion · Stroke · Neuroprotection · Neuroinflammation · Oxidative Stress

Introduction

Ischemic stroke is the second leading cause of death and the first major cause of disability in the world (Donkor 2018). Cerebral ischemia occurs when the blood flow of the entire or the major parts of the brain is disturbed, leading to cerebral tissue damage due to oxygen and glucose deprivation (Ostrowski et al. 2016). Cerebral ischemia–reperfusion (I/R) injury refers to restoring blood flow after a period of cerebral ischemia, which exacerbates neuronal injury (Sun et al. 2016). Reducing brain injury induced by I/R is an essential challenge in medicine. Neural injuries as a result of cerebral I/R increase the mortality and morbidity rates as well as care costs. Several pathophysiological pathways are
triggered during cerebral I/R, including neuroinflammatory and oxidative stress, cellular toxicity, ionic pump dysfunction, excitotoxicity, and nitric oxide release pathways. These pathways eventually lead to neuronal injury and apoptosis, especially in CA1 hippocampus region (Giuliani et al. 2006; Badr et al. 2016; Vandresen-Filho et al. 2015). Previous studies reported that attenuation of these pathophysiological processes, increasing in Brain-Derived Neurotrophic Factor (BDNF) expression, and an improvement of cerebral metabolism could reduce cerebral injuries induced by I/R (Ang and Gomez-Pinilla 2007; Liebelt et al. 2010; Zhang et al. 2010). Transcranial direct current stimulation (tDCS), is a novel non-invasive neuromodulation treatment (Rahman et al. 2013), in which a weak electrical direct current is transmitted on the skull through the scalp in order to affect the neural activities. This treatment method is increasingly under investigation in basic and clinical research. The tDCS can manipulate the synaptic transmissions by changing the balance between glutamate and Gamma-aminobutyric acid (GABA) (Hunter et al. 2015) concentrations and modify the functional connectivity between different regions of the brain (Fonteneau et al. 2018). It is demonstrated that N-Methyl-D-aspartate (NMDA) receptors activity is regulated by tDCS (Kim et al. 2010). These currents are also involved in changing the ionic concentration, proteins passing the membrane, and hydrogen ions (Clark et al. 2011). Resting membrane potential changes are also induced by tDCS. The tDCS manipulates the excitability and activity of neural and non-neural cells (Philip et al. 2017; George and Aston-Jones 2010) such as endothelial cells, lymphocytes, microglia and astrocytes, which eventually leads to neuroinflammation and oxidative stress modulation (Gellner et al. 2016; Monai et al. 2016). Furthermore, the pathways involved in neural rehabilitation are activated by these currents (Kang et al. 2013; Yu et al. 2018; Yoon et al. 2016; Merzagora et al. 2010). Today, treatments are designed to eliminate ischemia causes, and also supportive procedures are performed during the reperfusion period. In previous studies, single anodal or cathodal currents were used during the reperfusion period. However, the effects of these currents have not been investigated during ischemia stage and combined modes. In this study, we investigated the effects of using single and combined cathodal and anodal direct current to weaken pathophysiological pathways and reduce neuronal injury induced by I/R.

Material and method

Animals

Ninety male Wistar rats weighing 300–350 g were used and kept in standard cages with a controlled temperature of 22 ± °C, a 12:12-h light/dark cycle, and allowed free access to food and water throughout the study. The sample size of this study was estimated based on the results of similar articles. All procedures were approved by the ethics committee of the Tehran University of Medical Sciences (No: IR.TUMS.MEDICINE.REC.1399.643).

Study groups

The animals were randomly divided into six groups (n = 15): sham, I/R, I/R + a-DCS, I/R + c-tDCS, I/R + a/c-tDCS, and I/R + c/a-tDCS. In the sham group, rats were intact (no stimulation and cerebral I/R). In the I/R group, rats were subject to cerebral ischemia followed by reperfusion. In the I/R + c-tDCS and I/R + a-tDCS groups, the cathodal and anodal currents were singly applied, respectively, in both cerebral I/R periods. In the I/R + c/a-tDCS group, which are introduced as combination mode, the cathodal current (in the ischemia stage) and anodal current (in the reperfusion period) were applied. In the a/c-tDCS group (as combination mode), the anodal and cathodal currents were applied during the ischemia and reperfusion, respectively (Fig. 1). The mortality rate of experimental animals was expressed in Table 1.

Cranial electrode placement

Rats were anesthetized by an intraperitoneal injection of ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg), one day before ischemia induction. Then the rats were placed in a stereotaxic device. After disinfecting the scalp, the skin was cut to show the surface of the rat skull. Two holes were made in the cranium bone using a dental drill, and two screws were implanted in the skull as a pillar. The active (cranial) electrode was placed between Lambda and Bregma points and then fixed to the skull by anchoring screws and dental cement.

Induction of global cerebral ischemia

Four-vessel occlusion (4-VO) model was used to induce global cerebral I/R (Sun et al. 2009). Simultaneously with electrode placement and after placing the rats in the stereotaxic device, the posterior area of the rat’s neck was disinfected with betadine 5%. Then the skin and muscles along the posterior midline were cut. Two vertebral arteries, located under the alar foramen in the first cervical vertebra, were cauterized using electrocoagulation to permanently occlude the arteries, then the skin was sutured. After the animal’s recovery, they were returned to their cages. The next day, the rats were anesthetized again by an intraperitoneal injection of ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg) and positioned in the supine position. An incision was made along the anterior
neck by a scalpel. The fascia and muscles were dissected to expose the carotid arteries. The arteries and vagus nerves were isolated from each other. Both common carotid arteries were simultaneously blocked by microvascular clamps for 15-min in the ischemia stage. The micro-clamps were then opened to restore blood flow, in the reperfusion stage (five days). The disappearance of brain waves (Fig. 2), dilated pupils, and absence of corneal reflex were assessed to confirm cerebral I/R. Animal’s body temperature was monitored during the surgery.

**Table 1** Mortality rate of study animals

| Groups            | Mortality rate |
|-------------------|----------------|
| Sham              | 0              |
| I/R               | 6              |
| I/R + c-tDCS      | 4              |
| I/R + a-tDCS      | 5              |
| I/R + a/c-tDCS    | 4              |
| I/R + c/a-tDCS    | 2              |

**Fig. 1** Study design

**Fig. 2** EEG abnormalities during cerebral I/R
and maintained at 37 °C by a heating blanket. After their complete recovery, the rats were transferred to their cages again.

Transcranial direct current stimulation

The cranial electrode was a 1-cm diameter and circular shape conductor with a contact area of 0.785 cm². The counter electrode, which is composed of a large conventional rubber plate electrode (3 × 3.5 cm, Cross section = 10.5 cm²), was placed onto the rat’s ventral thorax. Experimental animals received a constant current intensity of 0.4 mA by tDCS device (CX 6650, Schneider Electronics, Gleichen, Germany), 15 min during the ischemia period, and then 30-min sessions twice a day up to 5 consecutive days, in five days of reperfusion period. In the anodal stimulation, the positive pole of the tDCS device was connected to the cranial active electrode and the negative pole to the counter electrode. In the cathodal stimulation, the negative pole of the device was connected to the active cranial electrode and the positive pole to the counter electrode. According to current’s duration and amount, the values of charge density and current density were below the neural damage threshold and within the reported limits of safety and tolerability (Peruzzotti-Jametti et al. 2013). No side effects related to using tDCS were seen in the study samples.

Tissue assessment

On the sixth day of I/R, the rats were anesthetized by an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). The animal’s head was severed by a guillotine. The hippocampal tissue was quickly isolated, weighed, and homogenized (10% w/v) in cold PBS (0.1 M, pH 7.4). The homogenates were centrifuged at 1.2 × 10^4 g at 4 °C for 20 min. The supernatants were stored at −70 °C. Tissue samples were used to determine the levels of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), malondialdehyde (MDA), superoxide dismutase (SOD), nitric oxide synthase (NOS), and N-Methyl-D-aspartate receptor (NMDAR). Protein levels in hippocampal homogenates were evaluated via Bradford technique. Hematoxylin–Eosin (H/E) and Nissl staining of the hippocampal CA1 region were evaluated for histological assessment and neuronal death. Cerebral edema was also measured.

IL-1β and TNF-α measurements

According to the company guidance, the levels of IL-1β and TNF-α were determined using a Rat-special sandwich enzyme-linked immunosorbent assay (ELISA) kit. All ELISA tests were performed in duplicate. The rat IL-1β and TNF-α particular monoclonal antibodies were put on microplates. The mix of homogenate, streptavidin-HRP, and biotinylated monoclonal antibodies were pipetted to the wells, and then a TMB substrate solution was added to the wells. The stop solution changed the colour from blue to yellow. The optical density of each well was immediately evaluated at 450 nm wavelength by an ELISA reader.

SOD and MDA measurements

MDA is a lipid peroxidation indicator and was measured by thiobarbituric acid reaction to produce the MDA-TBA adduct. The MDA level was colorimetrically quantified via a microplate reader at a wavelength of 530 nm. The SOD activity of hippocampus homogenates was measured based on the kit factory instruction. Homogenate samples were added to the wells then WST-1 working solution and enzyme working solution were also added, and incubated for 20 min at 37 °C. The SOD level was colorimetrically quantified via a microplate reader at a wavelength of 405 nm.

NOS measurements

Nitric Oxide (NO) level was determined indirectly by NOS activity. NOS activity assay kit provides an accurate method to assay NOS activity in various samples. In this method, NO generated by NOS underwent a series of reactions and reacted with Griess Reagent 1 and 2 to develop a colored product with strong absorbance at OD 540 nm.

NMDAR measurement

The level of NMDAR1 was measured using a Rat ELISA kit. All ELISA tests were performed in duplicate. Homogenate hippocampus samples and NR standard antibodies were added to the wells. Furthermore, biotinylated Rat NR antibody liquid and enzyme-conjugated fluid were added to every well. Then TMB development solution was added and catalyzed by HRP, generating a blue coloration. After adding a stop solution, the coloration was changed from blue to yellow. The samples were evaluated by an ELISA reader at 450 nm wavelength.

Brain edema measurement

Seven rats were anesthetized, killed, and their brain was removed. The olfactory bulb and pons were isolated then brain weight was measured using a scale (wet weight, WW). Then the brain was kept in an oven at 110 °C for 24 h and weighed again (dry weight, DW). The percentage of cerebral edema was calculated by a formula.

\[
\text{Edema} = \frac{\text{WW} - \text{DW}}{\text{WW}} \times 100
\]
Sensory and locomotor functions measurement

A hot plate and an open field tests were used to evaluate sensory and locomotor disorders induced by cerebral I/R. The hot plate was used to detect sensory defects. The paw licking time was measured when the rats were placed on a hot plate at 50 °C. The open field test was used to assess the locomotor activity of rats. In this test, a square box made of PVC with dimensions 60 × 60 × 30 cm was used. The area inside this box includes 36 houses of 10 cm. Seven rats were placed in a box for one minute to move freely. One hour later, the rat was again placed in the middle of the box for 5 min to move. The number of the houses that rats entered using all four hands and feet were counted.

Histological assessment and staining

On the sixth day after I/R, three rats’ brain was fixed with 4% paraformaldehyde. Histopathological assessment and dead neurons count in the CA1 hippocampal region were assessed using H/E and Nissl staining.

Statistical analysis

Data were assessed using the GraphPad prism 8. All data are described as mean ± Standard error of the mean (SEM). The study data had a normal distribution so parametric tests were used for analysis. The comparison among groups was analyzed using a one-way analysis of variances (ANOVA) followed by Tukey’s post-hoc. P-values < 0.05 considered statistically significant.

Results

The effects of tDCS on TNF-α and IL-1β levels

Our data showed the cerebral I/R caused an increase in levels of TNF-α and IL-1β in the hippocampal region compared to the sham group (P < 0.0001). Statistical analysis by one-way ANOVA revealed that there was a significant difference in levels of IL-1β and TNF-α between groups [F(5, 24) = 29.9, P < 0.0001 and F(5,24) = 43.7, P < 0.0001, respectively] (Fig. 3A-B). The level of IL-1β significantly declined in all groups receiving tDCS (P < 0.0001; Fig. 3A). In the c/a-tDCS, the decrease in IL-1 level was higher than other tDCS, and there was no significant difference with the sham group (P > 0.05). All tDCS significantly decreased the level of TNF-α in comparison to the I/R group (P < 0.0001; Fig. 3B). However, Tukey’s test revealed the reduction in the TNF-α level was considerably higher in the c/a-tDCS compared to the c-tDCS and a/c-tDCS (P < 0.0001, P < 0.01, respectively).

The effects of tDCS on SOD and MDA levels

Global cerebral I/R caused a significant increase in MDA level while decreased SOD level in the hippocampal region compared to the sham group (P < 0.0001, P < 0.0001,
respectively). One-way ANOVA indicated that there was a significant difference in the MDA and SOD levels between groups \( F(5, 24) = 48.9, P < 0.0001 \) and \( F(5,24) = 56, P < 0.0001 \), respectively) (Fig. 4A-B). The MDA level significantly decreased among groups receiving tDCS in comparison to the I/R group (\( P < 0.0001 \), Fig. 4A); furthermore, a significant decrease showed in the c/a-tDCS group than the c-tDCS and a/c-tDCS groups (\( P < 0.05, P < 0.05 \), respectively), and there was no significant difference with the sham group (\( P > 0.05 \)). All tDCS modes significantly enhanced the SOD level than the I/R group (\( P < 0.0001 \), Fig. 4B). However, the c/a-tDCS caused a considerably more increase in SOD level in comparison to the a-, c-, and a/c-tDCS groups (\( P < 0.05, P < 0.0001 \), and \( P < 0.001 \), respectively; Fig. 4B).

The effects of tDCS on NOS level

Cerebral I/R significantly enhanced the level of NOS tissue in comparison to the sham group (\( P < 0.0001 \)). One-way ANOVA showed that there was a significant difference in the NOS level between groups \( F(5, 24) = 16, P < 0.0001 \) (Fig. 5). Tukey's analysis revealed using a/c-, a-, and c/a-tDCS significantly decreased the hippocampal NOS level in comparison to the I/R group (\( P < 0.05, P < 0.01, P < 0.0001 \)). A decrease in the hippocampal NOS activity was a relatively greater in the c/a-tDCS than the other stimulation groups.

The effect of tDCS on NMDAR level

Our data revealed a considerable increase in the hippocampal NMDAR content induced by global cerebral I/R in comparison to the sham group (\( P < 0.0001 \)). One-way ANOVA showed that there was a considerable difference in the NMDAR level following cerebral I/R, especially in the c/a-tDCS and an exception in the c-tDCS. $ P < 0.0001 $ vs. the sham group. # $ P < 0.05 $, ## $ P < 0.01 $, and ### $ P < 0.001 $ vs. the I/R group. ns: $ P > 0.05 $ no statistically significant difference. (\( n=5 \))

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**Fig. 4** The effects of tDCS on SOD and MDA levels in the hippocampal area. The statistical analysis was done using a one-way ANOVA test. Data are expressed as means±SEM of each group. All tDCS modes caused a significant reduction in oxidative stress following cerebral I/R, especially in the c/a-tDCS. $ S P < 0.0001 $ vs. the sham group in Fig. 4A-B. # $ P < 0.0001 $ vs. the I/R group in Fig. 4A-B. * $ P < 0.05 $ vs. the I/R+c/a-tDCS in Fig. 4A. * $ P < 0.05 $, ** $ P < 0.001 $, and *** $ P < 0.0001 $ vs. the I/R+c/a-tDCS group in Fig. 4B. ns: $ P > 0.05 $ no statistically significant difference. (\( n=5 \))

**Fig. 5** The effect of tDCS on NOS level in the hippocampal area. The statistical analysis was done using a one-way ANOVA test. Data are expressed as means±SEM of each group. All tDCS modes caused a significant reduction in NOS level following cerebral I/R, especially in the c/a-tDCS and an exception in the c-tDCS. $ S P < 0.0001 $ vs. the sham group. # $ P < 0.05 $, ## $ P < 0.01 $, and ### $ P < 0.001 $ vs. the I/R group. ns: $ P > 0.05 $ no statistically significant difference. (\( n=5 \))
between groups [$F(5, 24) = 25.5, P < 0.0001]$ (Fig. 6). Tukey’s analysis showed using a/c-tDCS and c-tDCS significantly decreased the hippocampal NMDAR content in comparison to the I/R group ($P < 0.0001$). The c-tDCS decreased significantly NMDAR content in comparison to the a-tDCS and c/a-tDCS groups ($P < 0.01, P < 0.05$, respectively). Furthermore, the a/c-tDCS reduced significantly NMDAR level than the a-tDCS and c/a-tDCS groups ($P < 0.01, P < 0.05$, respectively; Fig. 5).

**The effect of tDCS on cerebral edema**

Our results showed the cerebral edema induced by cerebral I/R in comparison to the sham group ($P < 0.0001$). One-way ANOVA revealed that there was a considerable difference in the cerebral edema between groups [$F(5, 36) = 43.4, P < 0.0001$] (Fig. 7). All tDCS significantly decreased the cerebral edema in comparison to the I/R group, the exception in the a-tDCS. In the a-tDCS, cerebral edema was significantly higher in comparison to the c-tDCS and a/c-tDCS ($P < 0.05$). In the c-tDCS and a/c-tDCS groups, the cerebral edema percentage was comparable with the sham group ($P > 0.05$).

**The effect of tDCS on sensory function**

The results of the hot plate test showed that the paw licking time was significantly increased in the I/R group compared to the sham group ($P < 0.0001$), indicating that the impaired sensory perception as a result of cerebral I/R injury. A one-way ANOVA test indicated that there was a considerable difference in the paw licking time among all groups [$F (5, 36) = 41.1, P < 0.0001$] (Fig. 8). Using different tDCS modes improved the impaired sensory perception induced by cerebral I/R ($P < 0.0001$), especially in the c/a-tDCS. Tukey’s test indicated improvement in the sensory perception was significantly higher in the c/a-tDCS than other groups receiving tDCS ($P < 0.05$; Fig. 8) and was comparable with the sham group ($P > 0.05$).
The effects of tDCS on locomotor activity

Our data showed that the global cerebral I/R caused a decrease in the locomotor activity than the sham group ($P < 0.0001$). A one-way ANOVA analysis showed that there was a significant difference in the locomotor activity between groups [$F(5,36) = 38.7, P < 0.0001$] (Fig. 9). Tukey’s test indicated tDCS meliorated the impaired locomotor activity as a result of cerebral I/R in comparison to the I/R group ($P < 0.01$). The increase in locomotor activity was significantly higher in the c/a-tDCS compared to the c-tDCS and a/c-tDCS groups ($P < 0.05$ and $P < 0.01$; respectively).

Histological assessment

Histological assessment was performed in the CA1 hippocampal region. H/E staining assessment showed that cerebral I/R caused neuronal tissue cohesion loss and degenerative changes (condensed nuclei with deeply colored, shrunken cell body, and augmented gaps) compared to sham group ($P < 0.0001$; Fig. 10). A one-way ANOVA analysis revealed that there is a statistically significant difference in neuronal death in the study groups [$F(3,12) = 126.9, P < 0.0001$] (Fig. 10). All tDCS polarities significantly decreased neural death induced by cerebral I/R, especially in the c/a-tDCS mode.

Nissl staining showed the dead neuronal cells in the CA1 hippocampal region (Fig. 11). Global cerebral I/R caused a significant increase in necrotic neurons compared to the sham group ($P < 0.0001$). A one-way ANOVA test indicated that there was a significant difference in the number of dead neuronal cells between groups [$F(5,12) = 63.2, P < 0.0001$] (Fig. 11). All tDCS treatments significantly decreased neuronal death induced by cerebral I/R in comparison to the I/R group ($P < 0.01$). In the c/a-tDCS, the neuronal death was significantly lower than the a-tDCS and c-tDCS groups ($P < 0.001$). Furthermore, necrotic neuronal death was considerably lower in the a/c-tDCS than a-tDCS group ($P < 0.05$).
Cerebral I/R can lead to apoptosis and neuronal death, particularly in the hippocampal region. We observed that tDCS modulated the increase in activity of pathophysiological pathways induced by I/R, especially in the combined modes.

Based on the results of our study, different polarities of tDCS decreased neuroinflammatory and oxidative stress induced by cerebral I/R, especially in the c/a tDCS mode (Figs. 3, 4 and 5). The previous study has demonstrated that oxidative stress and neuroinflammation pathways modulation by tDCS is still unknown (Guo et al. 2020). Glial cells and astrocytes activities increase rapidly following hypoxia condition and cerebral injury. This exacerbates neuronal injury by increasing the production of pro-inflammatory cytokines such as TNF-α and IL-1β, and reactive oxygen species (ROS) (Chamorro et al. 2016; Godinho et al. 2018). The tDCS can hinder ROS activity elevation in the hippocampus by increasing glutathione peroxidase expression (Leffa et al. 2018). It was shown that direct currents could alter microglia activity (Peruzzotti-Jametti et al. 2013; Zhang et al. 2020; Jung et al. 2020). The voltage ionic
channels of the microglia cells membrane are sensitive to electrical currents (Gellner et al. 2016). Changes in cells membrane polarity and potential as well as cell activity induced by tDCS are the outcomes of sodium and potassium uptake and excretion in the extracellular environment (Jackson et al. 2016). In addition to hyperpolarization of astrocytes and microglia, and their activity reduction, cathodal current can also induce negative feedback in NO synthase by increasing osteopontin (OPN) expression. OPN also plays a key role in regulating immune activity (Rabenstein et al. 2019). The protective effects can be related to BDNF expression stimulation by anodal tDCS, which ultimately lead to oxidative stress and inflammatory responses modulation, attenuation of mitophagy, and mitochondrial activity adjustment (Fritsch et al. 2010; Lee et al. 2019). BDNF triggers TrkB via several downstream signaling pathways containing CaMK, AKT, Raf / Ras / MEK / ERK, leading to cell survival (Alam et al. 2016). The BDNF can also reduce the activity and proliferation of microglia. Direct currents develop the proliferation and migration of nerve stem cells (NSCs) to damaged nerve areas. NSCs weaken neuroinflammatory processes and remove dead nerve tissues

![Fig. 11 Nissl staining of the CA1 hippocampal region in different groups. The black arrow show the death and necrotic neurons. Statistical analysis was done using a one-way ANOVA test. Data are expressed as the means ± (SEM). Applying tDCS in all modes prevented neural cells from death following I/R in the CA1 hippocampal region. Dead neurons count was lower significantly in combined modes than the other modes. $P<0.0001$ vs. the sham group. *$P<0.01$, **$P<0.001$, and ***$P<0.0001$ vs. the I/R group.

* $P<0.001$ vs. the I/R + c/a-tDCS. ¥ $P<0.05$ vs. the I/R + a-tDCS. ns: $P>0.05$ no statistically significant difference, (n=3). Scale bar=100 μm.](image)
in damaged neural areas (Rueger et al. 2012; Martino et al. 2011). Reducing neurons stress and modulating cells activity in the early stages of ischemia by applying cathodal current can play an important role in reducing neuronal damage, as well as increasing the activity of pathways such as BDNF by using anodal current causes stronger neuroprotective effects.

Our results showed that NMDA receptor level was remarkably decreased in c-tDCS and a/c-tDCS groups in the hippocampal region (Fig. 6). A previous study reported that anodal and cathodal currents could increase and decrease the NMDA level, respectively (Peruzzotti-Jametti et al. 2013). Cathodal direct current reduces nerve cells firing by reducing glutamate secretion and its receptors activity (Nitsche et al. 2003; Stagg and Nitsche 2011). Reducing neuron excitability and non-neural cell activities by cathodal currents in cerebral ischemia stress conditions can reduce oxygen and blood demands. This current also weakens pathological pathways induced by NMDA receptor stimulation in the acute phase. It was revealed that the after-effects of tDCS related to NMDA receptor adjustments (Nitsche et al. 2009). An increase in NMDA receptors activity for at least 24 h after primary brain injury can improve neurological and behavioral function (Shohami and Biegon 2014), which is consistent with the study results in improving sensory and motor functions. In c/a-tDCS group of our study, anodal stimulation was applied 24 h after ischemia induction. The elevation in glutamate level and NMDA receptor activity increase the neurological injury in the acute injury phase; on the other hand, it also, improves learning and sensory-motor functions in the recovery phase.

Our results showed that cerebral edema was significantly reduced when c-tDCS and a/c-tDCS modes were used (Fig. 7). Cerebral edema occurs as a result of lack of oxygen and energy in nerve cells, dysfunction of membrane pumps and ionic imbalance. It was reported that using drugs or methods to improve ionic channels function, reduction of inflammatory, oxidative stress and apoptosis can play a significant role in enhancing blood–brain barrier (BBB) function and reducing cerebral edema. (Yao et al. 2020). Other studies showed that c-tDCS could reduce edema and brain damage by reducing inflammatory responses and free radicals (Peruzzotti-Jametti et al. 2013; Liu et al. 2017). It seems that using multiple sessions of cathodal currents can be more effective in reducing cerebral edema. A previous study reported that using anodal current in middle cerebral artery occlusion exacerbated cerebral edema (Peruzzotti-Jametti et al. 2013). Anodal current reduces tight junctions and vascular tone in brain’s blood vessels. In the reperfusion phase, cerebral blood flow elevation can increase cerebral edema development by exacerbating oxidative stress and BBB disorder (Peruzzotti-Jametti et al. 2013). Ionic changes in cells including sodium, chloride, and water play an important role in cytotoxic edema (Yao et al. 2020). In addition to modulating astrocytes and glial cells activities, direct currents can affect the activity of ionic channels and the regulation of ionic concentrations such as sodium, chloride, and potassium. A diminish in AMPA receptor and aquaporin-4 channels activities may also play a role in edema reduction. Using cathodal current can induce transmission, phosphorylation, and changes in the activity of AMPA receptors (Stafford et al. 2018). Reducing AMPA receptor activity and using its antagonist can effectively reduce cerebral edema (Atsumi et al. 2003).

Our results showed that using different polarities of tDCS could improve sensory and locomotor dysfunctions induced by cerebral I/R (Figs. 8 and 9). Notably, the performance improvement was higher in the c/a-tDCS mode than in other modes. Direct currents can change the activity of sensory-motor cortical regions via glutaminergic and GABA pathways (Di Lazzaro and Rothwell 2014). The tDCS can alter the function of motor areas via the excitability of neural networks and plasticity (Morya et al. 2019). Immediate effects of anodal and cathodal stimulation induce the excitability elevation and reduction in the sensory-motor cortex, respectively. The previous study showed that using anodal tDCS could improve motor function after brain injury, and had protective effects against cerebral degenerative changes (Kim and Han 2017), however, using cathodal current didn’t have a role in motor rehabilitation (Zhang et al. 2020). Previous studies were further investigated tDCS in rehabilitation phase (O’Brien et al. 2018). Induction of subthreshold depolarizations by anodal direct current reduced the level of cortical injury and excessive inhibitory signals resulting from cerebral ischemia injury (Sanchez-Leon et al. 2021). Another study revealed that anodal tDCS stimulated the activity of dopaminergic pathways and M1 region, and boosted motor functions (Lu et al. 2015; Li et al. 2015). Polania et al. (2011) showed that using anodal tDCS could cause more and stronger neuronal connections within and among the cerebral and between the frontal and peritoneal lobes, and also improved the functions of sensory-motor networks in the brain. In the a-tDCS and c/a-tDCS groups (anodal current in the reperfusion period), we found more improvement in the recovery of sensory and locomotor than the other stimulation groups.

Histological assessment results showed that using tDCS during I/R phases reduced neuronal death, especially in c/a-tDCS mode. Nissl and H/E staining observations (Figs. 10 and 11) are consistent with the other findings of oxidative stress and inflammatory markers. According to previous studies, tDCS decreased microglia activity, and eventually reduced apoptosis and cell death (Peruzzotti-Jametti et al. 2013; Villa et al. 2010). Baba et al. (2009) stated that electrical stimulations have anti-apoptotic functions via phosphoinositide 3-kinase/Akt signaling pathway. Another research stated that the autophagy and
neuroprotective effects of tDCS might be related to mTOR expression (Podda et al. 2016; Chen et al. 2013; Smith et al. 2014). One of cerebral ischemia–reperfusion injury pathways is mitochondrial dysfunction. Anodal direct current reduces mitochondrial edema and mitochondrial dehydrogenase activity, and also this current contributes to biogenesis, mitophagy attenuation, and mitochondrial dynamics by modulating fusion and fission processes (Lee et al. 2018). Modulating inflammatory and oxidative stress responses reduces apoptosis and neuronal death in cerebral hypoxia conditions. According to the previous studies and the results of the present study, the activity of responsible cells in inflammatory and oxidative stress production can be modulated by different types of direct currents (Rueger et al. 2012). The cathodal current can effectively reduce injuries by reducing pathological factors during cerebral ischemia, but anodal stimulation augments the cerebral recovery process.

In the previous studies, only a single direct current was used, but in our study, both currents were applied in different stages of I/R. In the previous studies, the goals were more on rehabilitation. However, in our study, tDCS was applied simultaneously in I/R phases.

**Conclusion**

Using cathodal and anodal tDCS in both cerebral I/R phases can reduce neuronal injury in the hippocampal region, especially in the c/a-tDCS mode. Furthermore, due to the importance of therapeutic time in cerebral ischemia injury, immediate application of these currents simultaneously with ischemia and its following reperfusion stage can have more neuroprotective properties and improve cerebral recovery.

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**Author contributions** Karimian and Kaviannejad designed the subject and plan of this study; Karimian, Riahi and Kaviannejad collaborated in study design and experiments; Kaviannejad and Ashabi performed animal experiments; Riahi and Karimian performed data analysis. All authors whose names emerge on the submission approved the version to be published.

**Data availability** The authors confirm that all data generated or analyzed during this study are included in this published article.

**Declarations**

**Ethical approval** All animal procedures and experimental protocols were approved by Ethics Committee of TUMS (No: IR.TUMS.MEDICINE.REC.1399.643).

**Conflicts of interest** The authors declare that they do not have conflict of interest.

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