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 neural injuries by using both transcranial direct current stimulation (tDCS) polarities in cerebral ischemia and its following reperfusion periods. 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 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-1 β , 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-1 β 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.

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 damages 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 (Giuliani et al. 2006; Badr et al. 2016). The neurons of the hippocampal CA1 region are highly

susceptible to ischemia (Vandresen-Filho et al., 2015). Previous studies reported pathophysiological process attenuation, increasing in neurogenesis and Brain-Derived Neurotrophic Factor (BDNF) expression, and an improvement of cerebral metabolism that reduced 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), that transmits a weak direct current on the skull through the scalp, therefore this treatment method is increasingly under investigation in basic and clinical researches. The tDCS can manipulate the synaptic transmissions by changing the balance between glutamate and Gamma-aminobutyric acid (GABA) (Hunter et al. 2015) concentration 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 was 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 were 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, microglial 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, 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 300–350g were used and kept in standard cages, that includes a controlled temperature of $22\pm^{\circ}\text{C}$, a 12:12-h light/dark cycle, and allowed free access to food and water throughout the study. 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 sham group, rats were intact (no stimulation and cerebral I/R). In I/R group, rats were subject to cerebral ischemia followed by reperfusion. In 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).

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 cranial using a dental drill, and two screws were implanted in the skull as a pillar. The active electrode was placed between Lambda and Bregma points 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 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 and positioned in 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 micro-vascular clamps for 15-min in the ischemia stage. The micro-clamps were then opened to restore blood flow, in the reperfusion stage. The disappearance of brain waves, dilated pupils, and absence of corneal reflex were assessed to confirm cerebral I/R. Animal's body temperature was monitored during the surgery 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 active electrode was a 1-cm diameter, circular shape with a contact area of 0.785 cm². The counter electrode, which is composed of a large conventional rubber plate electrode (3×3.5cm, Cross section=10.5cm²), was placed onto the rat's ventral thorax. Experimental animals received a constant current intensity of 0.4 mA by tDCS (CX 6650, Schneider Electronics, Gleichen, Germany), 15 minutes during the ischemia period, and then 30-minute sessions twice a day up to 5 consecutive days, in the reperfusion period. 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). 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.

Tissue assessment

On the sixth day of I/R, the rats were decapitated. 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 1.2×10^4g 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-1 β (IL-1 β), 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. A Hot plate and an open field tests were performed to evaluate the sensory and motor performances.

IL-1 β and TNF- α measurements

According to the company guidance, the levels of IL-1 β and TNF- α were determined using a Rat-special sandwich enzyme-linked immunosorbent assay (ELISA) kit. The rat IL-1 β and TNF- α particular monoclonal antibodies were put on microplates. The mix of homogenate, streptavidin-HRP, and biotinylated monoclonal antibodies was 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.

NMDA measurement

The level of NMDAR1 was measured using a Rat ELISA kit. 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

The 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 hours and weighed again (dry weight, DW). The percentage of cerebral edema was calculated by a formula.

$$Edema = \frac{ww - dw}{ww} \times 100$$

Sensory and locomotor function measurement

A Hot plate and an open field tests were used to evaluate sensory and locomotor disorders induced by the 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. The rat was 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 minutes 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, rats' brain was fixed with 4% paraformaldehyde. Histopathological assessment and dead neurons in the CA1 hippocampal region were assessed using H/E and Nissl staining.

Statistical analysis

Data were assessed using the GraphPad prism. All data are described as mean ± Standard error of the mean (SEM). The comparison among groups was analyzed using a one-way analysis of variances (ANOVA) followed by Tukey's *post-hoc*. P-values the findings <0.05 are statistically remarkable.

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 [$F(5, 24) 29.9, p < 0.0001$; Fig.2A. $F(5,24) 43.7, p < 0.0001$; Fig.2B, respectively]. The level of IL-1β significantly declined in all groups receiving tDCS ($p < 0.0001$; Fig.2A). 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.2B). 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).

Figure 2. The effects of tDCS on levels of IL-1 β and TNF- α in the hippocampal region. Data are expressed as means $\pm$ SEM of each group. The statistical analysis was done using a one-way ANOVA test. All tDCS modes caused a significant reduction in neuroinflammation following cerebral I/R, especially in the c/a-tDCS. \$ $p < 0.0001$ vs. the sham group. $\neq$ $p < 0.0001$ vs. the I/R group in fig 2A and 2B. * $p < 0.01$ and ** $p < 0.001$ vs. the I/R+c/a-tDCS group in fig.2B. ns: $p > 0.05$ no statistically significant difference. (n=5).

The effects of tDCS on SOD and MDA levels

Global cerebral I/R caused a significant increase in MDA level while decrease 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, $p < 0.0001$; Fig.3A. F(5,24) 56, $p < 0.0001$; Fig.3B, respectively]. The MDA level significantly decreased among groups receiving tDCS in comparison to the I/R group ($p < 0.0001$, Fig.3A); 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.3B). 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.3B).

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.4]. Tukey's analysis revealed using a/c-, a-, and c/a-tDCS significantly decreased the hippocampal of NOS level in comparison to the I/R group ($p < 0.05$, $p < 0.01$, $p < 0.0001$). A decrease in the hippocampal of NOS level exposed to the I/R was a relatively greater reduction in the c/a-tDCS than other stimulation groups and there was no significant difference with the sham group ($p > 0.05$).

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 between groups* [F(5, 24) 25.5, $p < 0.0001$; Fig.5]. 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 content than the a-tDCS and c/a-tDCS groups ($p < 0.01$, $p < 0.05$, respectively; Fig.5).

Figure 5. The effects of tDCS on NMDAR content in the hippocampal region. The statistical analysis was done by using a one-way ANOVA test. Data are expressed as means $\pm$ SEM of each group. The c-tDCs and a/c-tDCS caused a significant decrease in NMDAR level following cerebral I/R. # $p < 0.0001$ vs. the sham group. # $p < 0.0001$ vs. the I/R group. * $p < 0.05$ and ** $p < 0.01$ vs. the I/R+c-tDCS group. ¥ $p < 0.05$ and ¥¥ $p < 0.01$ vs. the I/R+a/c-tDCS group. ns: $p > 0.05$ no statistically significant difference. (n=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, 12) 14.6, $p < 0.0001$; Fig.6]. 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 ($p > 0.05$)

Figure 6. The effects of tDCS on cerebral edema. The statistical analysis was done using a one-way ANOVA test. Data are expressed as means $\pm$ SEM of each group. All tDCS modes caused a significant decrease in the cerebral edema following cerebral I/R, an exception in the a-tDCS. \$ $p < 0.0001$ vs. the sham group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. the I/R group. ¥ $p < 0.05$ vs. the I/R+a-tDCS group. ns: $p > 0.05$ no statistically significant difference. (n=7)

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.7]. Using different tDCS modes improved the impaired sensory perception induced by cerebral I/R ($p < 0.0001$), especially in the c/a-tDCS method. 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.7) 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.8]. 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).

Figure 8. The effects of tDCS on locomotor activity. The statistical analysis was done using a one-way ANOVA test. Data are expressed as means $\pm$ SEM of each group. All tDCS modes caused a significant improvement in locomotor activity following cerebral I/R, especially in the c/a-tDCS. \$ $p < 0.0001$ vs. the sham group. # $p < 0.01$ and ## $p < 0.0001$ vs. the I/R group. * $p < 0.05$ and ** $p < 0.01$ vs. the I/R+c/a-tDCS group. ns: $p > 0.05$ no statistically significant difference. (n=7)

Histological assessment

H/E staining in the hippocampal CA1 region showed that a large population of neurons was observed alive with the regular arrangement in the sham group. The histological assessment showed that cerebral I/R caused neuronal tissue cohesion lost and degenerative changes (condensed nuclei with deeply colored, shrunken cell body, and augmented gaps), that indicates a remarkable neuronal death in the CA1 hippocampal region (50-60%) compared to sham group. Less neuron death was observed in the I/R+c-tDCS and I/R+a-tDCS groups compared to the I/R group. In c-tDCS and a-tDCS groups, the population of neural death (30-40%) increased compared to the a/c-tDCS and c/a-tDCS groups, and a relatively more reduction in neurons death was found in c-tDCS. In the I/R+c/a-tDCS and I/R+a/c-tDCS groups, the percentage of neuron death increased compared to the sham group. Less neuron death (10%) was observed in c/a-tDCS than a/c-tDCS.

Figure. Morphological assessment of neuron death by H/E staining in different groups. H/E staining shows the neurons death in the CA1 hippocampal region. The black arrows indicates the neuronal death with a condensed nucleus, deeply colored, and shrunken cell body. In the c/a-tDCS, lesser hippocampal neuronal death was observed than the other tDCS modes. (n=3) Scale bar=100 μ m

Nissl staining showed the dead *neuronal* cells in the CA1 hippocampal region (Fig.10). 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 dead neuronal cells between groups* [F (5, 12) 63.2, $p < 0.0001$; Fig.10]. 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 ($p < 0.05$).

Figure 10. Nissl staining of the CA1 hippocampal region in different groups. The black arrow shows the death and necrotic neurons. Statistical analysis was done using a one-way ANOVA test. Data are expressed as the means $\pm$ (SEM). All tDCS caused a lesser hippocampal neuronal death following cerebral I/R, especially in the combined 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 μ m.

Discussion

Cerebral I/R can lead to apoptosis and neuronal death, particularly in the hippocampal region. We observed that tDCS modulated the physiopathological pathways of the cerebral exposed to I/R, especially in the combined mode (c/a-tDCS).

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 (Fig. 2-4). 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 due to stress neurons in hypoxia conditions and cerebral injury. This exacerbates neuronal injury by increasing the production of pro-inflammatory cytokines such as TNF- α and IL-1 β , and reactive oxygen species (ROS) (Chamorro et al. 2016; Godinho et al. 2018). The tDCS can respond to ROS activity elevation in the hippocampus by increasing glutathione peroxidase expression (Leffa et al. 2018). It was shown that direct currents altered 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 in the acute and early stages of injury, cathodal current can 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 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 tissue in damaged neural areas (Rueger et al. 2012; Martino et al. 2011). Reducing stress neurons and modulating cell activity in the early stages of ischemia by applying cathodal current can play an important role in reducing neuronal damage and increasing the activity of neuroprotective pathways such as BDNF by using anodal current causes stronger synergistic effects.

Our results showed that NMDA receptor level was remarkably decreased in c-tDCS and a/c-tDCS groups in the hippocampal region (Fig. 5). A previous study reported that anodal and cathodal currents could increase and decrease the NMDA level, respectively 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 et al. 2011). Reducing neurons excitability and non neuronl cell activities by cathodal currents in stess ischemia 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 hours after primary brain injury can improve neurological and behavioral function (Shohami and Biegon et al., 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 hours after ischemia induction. The elevation in glutamate level and NMDA receptor activity increases 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. 6). Cerebral edema occurs as a result of lack of oxygen and energy in nerve cells, dysfunction of membrane pumps and ionic imbalance. In a previous study, it was reported that using drugs or methods that aim to improve ionic channels, anti-inflammatory, oxidative stress and anti-apoptotic properties effectiveness can play a significant role in enhancing blood-brain barrier (BBB) functioning 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, chlorine, 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 (Fig. 7-8). Notably, the performance improvement was higher in the c/a-tDCS mode than in other modes. Direct currents can effect on the sensory-motor cortical regions via glutaminergic and GABA pathways (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 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 improving motor rehabilitation (Zhang et al. 2020). Previous studies were further investigated in the 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 (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. (2013) 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 groups.

Histological results showed that using tDCS during I/R phases reduced neuronal death, especially in c/a-tDCS mode (Fig. 9-10). 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. This current also contributes in 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 hypoxia cerebral tissues. According to the previous studies and the results of the present study, the activity of responsible cells in inflammatory factors 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.

Nissl and H/E staining observations (Fig. 8-9) are consistent with the other findings of oxidative stress and inflammatory markers. In the previous studies, only a single direct current was used, but in our study, both currents were applied by using a new technique in different stages of I/R. In the previous studies, the goals were more on rehabilitation and initiation of tDCS in the reperfusion phase. 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 hippocampal, especially in the c/a-tDCS mode. Furthermore, due to the importance of therapeutic time in ischemia brain injury, immediate application of these currents simultaneously with ischemia and its following reperfusion stage can have more neuroprotective properties and improve cerebral recovery.

Declarations

Acknowledgments

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Author contributions

Karimian and kaviannejad designed the subject and scheme of this research; Karimian, Riahi and kaviannejad take part in the design of research and experiments; Kaviannejad and Ashabi take part in the animal experiments; Riahi and Karimian participated in result analysis. All authors whose names emerge on the submission approved the version to be published.

Compliance with ethical standards

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

Data availability

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

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Figures

Figure 1

Study design

Figure 2

The effects of tDCS on levels of IL-1 β and TNF- α in the hippocampal region. Data are expressed as means $\pm$ SEM of each group. The statistical analysis was done using a one-way ANOVA test. All tDCS modes caused a significant reduction in neuroinflammation following cerebral I/R, especially in the c/a-tDCS. \$ p < 0.0001 vs. the sham group. $\neq$ p < 0.0001 vs. the I/R group in fig 2A and 2B. * p < 0.01 and ** p < 0.001 vs. the I/R+c/a-tDCS group in fig.2B. ns: p > 0.05 no statistically significant difference. (n=5).

Figure 3

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 $\pm$ SEM of each group. All tDCS modes caused a significant reduction in oxidative stress following cerebral I/R, especially in the c/a-tDCS. \$ p < 0.0001 vs. the sham group in fig.3A and 3B. # p < 0.0001 vs. the I/R group in fig.3A and 3B. * p < 0.05 vs. the I/R+c/a-tDCS in fig.3A. * p < 0.05, ** p < 0.001, and *** p < 0.0001 vs. the I/R+c/a-tDCS group in fig.3B. ns: p > 0.05 no statistically significant difference. (n=5)

Figure 4

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 $\pm$ 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. \$ $p < 0.0001$ 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)

Figure 5

The effects of tDCS on NMDAR content in the hippocampal region. The statistical analysis was done by using a one-way ANOVA test. Data are expressed as means $\pm$ SEM of each group. The c-tDCS and a/c-tDCS caused a significant decrease in NMDAR level following cerebral I/R. # $p < 0.0001$ vs. the sham group. # $p < 0.0001$ vs. the I/R group. * $p < 0.05$ and ** $p < 0.01$ vs. the I/R+c-tDCS group. ¥ $p < 0.05$ and ¥ $p < 0.01$ vs. the I/R+a/c-tDCS group. ns: $p > 0.05$ no statistically significant difference. (n=5)

Figure 6

The effects of tDCS on cerebral edema. The statistical analysis was done using a one-way ANOVA test. Data are expressed as means $\pm$ SEM of each group. All tDCS modes caused a significant decrease in the cerebral edema following cerebral I/R, an exception in the a-tDCS. \$ $p < 0.0001$ vs. the sham group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. the I/R group. ¥ $p < 0.05$ vs. the I/R+a-tDCS group. ns: $p > 0.05$ no statistically significant difference. (n=7)

Figure 7

The effects of tDCS on sensory perception. The statistical analysis was done using a one-way ANOVA test. Data are expressed as means $\pm$ SEM of each group. All tDCS modes caused a significant improvement in sensory perception following cerebral I/R, especially in the c/a-tDCS. \$ $p < 0.0001$ vs. the sham group. # $p < 0.0001$ vs. the I/R group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the I/R+c/a-tDCS group. ns: $p > 0.05$ no statistically significant difference. (n=7)

Figure 8

The effects of tDCS on locomotor activity. The statistical analysis was done using a one-way ANOVA test. Data are expressed as means $\pm$ SEM of each group. All tDCS modes caused a significant improvement in locomotor activity following cerebral I/R, especially in the c/a-tDCS. \$ $p < 0.0001$ vs. the sham group. # $p < 0.01$ and ## $p < 0.0001$ vs. the I/R group. * $p < 0.05$ and ** $p < 0.01$ vs. the I/R+c/a-tDCS group. ns: $p > 0.05$ no statistically significant difference. (n=7)

Figure 9

Morphological assessment of neuron death by H/E staining in different groups. H/E staining shows the neurons death in the CA1 hippocampal region. The black arrows indicates the neuronal death with a

condensed nucleus, deeply colored, and shrunken cell body. In the c/a-tDCS, lesser hippocampal neuronal death was observed than the other tDCS modes. (n=3) Scale bar=100µm

Figure 10

Nissl staining of the CA1 hippocampal region in different groups. The black arrow shows the death and necrotic neurons. Statistical analysis was done using a one-way ANOVA test. Data are expressed as the means $\pm$ (SEM). All tDCS caused a lesser hippocampal neuronal death following cerebral I/R, especially in the combined 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.

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