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Evaluation of The Effect of Applying Anodal tDCS in The LPFC and RPFC Regions and 10x10, 25x25 and 50x50 Protocols on The Acquisition of Fear Extinction Memory and The Time Latency of The Activation of Fear Memory: An Animal Study of PTSD

Mohammad-Reza Vahed[1*], Mohammad Nasehia[2], Mohammad-Reza Zarrindast[3], Mahdieh Azizian[4,5]

[1]*Department of Medical Physics, Afzalipour School of Medicine, Kerman University of Medical Sciences, Kerman, Iran.*
[2]*Cognitive and Neuroscience Research Center (CNRC), Tehran Medical Sciences Branch, Islamic Azad University, Tehran, Iran*
[3]*Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.*
[4]*Modeling in Health Research Center, Institute For Futures Studies in Health, Kerman University of Medical Sciences, Kerman,Iran. 0000-0001-8149-5268*
[5]*Department of General Education, Afzalipour School of Medicine, Kerman University of Medical Sciences, Kerman,Iran. 0000-0001-8149-5268*

[*]***Corresponding authors Email:*** *mr_vahed@kmu.ac.ir*

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Abstract

tDCS *method is considered as the first clinical method for the treatment and control of many mental disorders, including PTSD. In treatment of PTSD, the first step is to use a psychotherapy method called PE, which is based on creating fear extinction memory. In this research, we investigated the effect of tDCS method with TD=18, LPFC and RPFC areas and 10x10, 25x25 and 50x50 protocols on the acquisition of fear extinction memory and the time latency the fear memory activation. 90 NMRI male mice were used in the age range of 9-11 weeks. The mice were divided into three groups A, B and C. Each group was classified into three subgroups: Control, LPFC and RPFC. Results: except for group A, in groups B and C, there is a significant difference between LPFC and RPFC subgroups and the control subgroup It was also shown that a change in the protocol for formation fear extinction memory can cause a significant change in the ability to acquisition fear acquisition memory. Also, these results showed that tDCS stimulation of the LPFC and RPFC regions can cause opposite and significant changes in the time delay of fear memory activation. Conclusion: training protocols with more repetition and anodal tDCS in the LPFC can strengthen the formation of fear extinction memory and increase the time latency in the activation of the fear memory in the test or retrieval phase, and anodal tDCS in the RPFC and the use of training protocols with less repetition can produce the opposite results. Due to the importance of the obtained results, please refer to the suggestions section of the article.*

Keywords: *Anodal tDCS, Acquisition, Fear Extinction memory, LPFC, RPFC, PTSD.*

1. Introduction

Experience or witnessing traumatic event can greatly affect a person's life and cause various mental disorders such as PTSD or various psychosomatic disorders [1]. PTSD is considered to be the most important mental disorder caused after a traumatic event and it can lead to other types of mental disorders such as depression which refers to deep stress and fear that can affect the whole life of the injured person [2-4]. A group of epidemiological studies has shown that about 70% of people have

experienced such traumatic events in different ways during their lives, of which about 4% have entered the stage of PTSD disorder, 2 to 3% of them until the end of their lives [2-7]. Today, two methods of psychotherapy and pharmacotherapy are used to treat and control this important disorder, and in most cases psychotherapy is used as the first treatment method and the front line of treatment and control of this disorder [8, 9]. The basis of psychotherapy is based on a method called PE (Prolong Experience), which is part of a group of psychotherapy methods called TF-CBT (Trauma-Focused Cognitive Behavior Therapy) [2, 8]. In the PE method, patient with PTSD are exposed to a specific symptom of a traumatic event for a long time in a safe and controlled context, so that the fear extinction memory is formed in the patient's brain [10]. This treatment strategy is implemented after activating the cognitive brain mechanisms of fear through fear conditioning (CS + US) by exposing the patient to the CS only for a long time [8, 11-13]. Due to the importance of PE method in the treatment of various mental disorders including PTSD, extensive research has been started for a long time in human and animal fields to investigate the factors affecting the different stages of fear extinction memory formation and its continuation [14]. In this regard, one of the most important research fields at present is the investigation of the effect of weak constant electric currents on the different stages of fear extinction memory formation using the tDCS method [11, 13, 15-18]. tDCS (transcranial Direct Current Stimulation) is a non-invasive method of applying weak constant electric fields and currents to different regions of the nervous system, especially the brain. I this method, weak electrical currents are applied to the brain through certain areas of the scalp and using special electrodes without the need for surgery and electrode implantation (DBS). Today, it has been proven that this type of currents can have a neuro-modulation effect on different areas of the brain, it can affect cognitive activities such as learning and memory [11, 15, 16, 19, 20]. In this study, the effect of Anodal tDCS, 10x10, 25x25 and 50x50 protocols and LPFC and RPFC regions on the acquisition of fear extinction memory and fear memory activation time latency in male mice were investigated and compared.

2. Material and method

90 NMRI male mice (35-30±3 g) in the age range of 9-11 weeks were used. Animals were kept in standard laboratory conditions (12 hours of light and 12 hours of darkness. Light starts at 7:00 AM and darkness at 8:00 PM) at a temperature of 20-25 ± 3 °C with free access to food and water (ad libitum). This research was carried out based on the guidelines published by the National Institute of Health (No.: 2010-23-83) and following the guidelines recommended by the Ethics Committee of Tehran University of Medical Sciences.

Surgery

In order to implant tDCS electrodes on the surface of the skull, rats were anesthetized using a combination of ketamine (50 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injection. The anesthetized mice were placed in the stereotaxic machine and a gap was made with a surgical blade in the surface of their skull under sterile conditions. LPFC and RPFC areas on the skull were precisely determined for the placement of electrodes using the Paximus and Franklin 2004 atlas and the stereotaxy system. After determining the Bragma point using atlases and the stereotaxic system, the location of the electrodes was determined as one millimeter in the ML or MR direction and one millimeter in the AP direction relative to the Bragma point, and the electrodes were placed on the skull using dental cement [21].

Transcranial direct current stimulation

tDCS device (Activtek Company-Taiwan) was used to apply weak electric constant current after checking the amount of instrumental error. Via the anodic electrode of the tDCS system, a current with characteristic TD=18 (0.6mA-30′) was applied to the rat brain in the LPFC and RPFC areas. The stimulation electrode was made of a plastic structure with a metal core, the inner diameter was 2.1 mm, and its contact surface with the skull was 3 mm^2. In order to fully connect the electrode with the surface of the skull, it is filled with 0.9% normal saline. The earth electrode was also made of carbon, which was placed in the abdominal thorax area of the mouse. Mice were immobilized using a special Plexiglas chamber during the experiment [22].

Conditioning system

In order to perform fear conditioning and fear forgetting, a light-proof and sound-proof chamber with dimensions of 55×53×67 cm^3 was used. This chamber was equipped with a 24-watt lamp, a speaker and three video cameras. For CS stimulation, this system could produce a sound with a frequency of 4 Hz and an intensity of 35 dB, and for US stimulation (unconditioned stimulus), a replaceable rod floor

that was equipped with a generator that could apply electric shocks with different intensity and frequency to stimulate US to the leg area of the mouse. In this study, a current intensity of 0.6 mA with a frequency of 50 Hz was used for US. In this system, it was possible to change the walls of the room to check the context effect [23].

Materials and Methods

Statistical Methods

Statistical analysis was performed using SPSS 15. Statistical analysis was performed by repeated measures (RM) one-way ANOVA followed by Tukey's and Scheffe's multiple comparisons test for post-hoc analysis. The reason for not presenting the results of the Bonferroni test was the complete similarity of its results with Tukey's test.

Design of experiments

In this study, 90 male mice were divided into three groups with protocols A (10 x 10), B (25 x 25) and C (50 x50) in such a way that 30 mice were placed in each group. Each group was divided into 3 subgroups: Control, LPFC, RPFC, and 10 male mice were included in each subgroup [24-27].

Fear conditioning

In the first stage, in each group, the mice of the subgroups were subjected to surgery and electrode implantation on the skull 5 days before the start of fear conditioning (for habituation and adaptation). Then the fear conditioning protocol was performed by applying CS+US on mice and 48 hours later, in the recall phase, the fear conditioning of mice was confirmed by applying only CS.

Fear extinction conditioning train

5 days after confirming the fear conditioning, the fear extinction conditioning protocol was implemented to formation of the fear extinction memory. Subgroups of LPFC and RPFC, except for the control group, were exposed to constant electric current using tDCS method before starting the training stage. At this stage, mice were only exposed to CS stimulation.

Fear extinction memory test

24 hours later in the test phase (retrieval phase) by repeating the training phase protocol by recording the time of freezing the mice and the latency time of activation of the fear memory, the effect of applying tDCS and using the different protocols was evaluated.

3. Results and Discussion

Group A (10x10)

The effect of anodal tDCS with TD=18 in the LPFC and RPFC regions on the amount of fear extinction memory acquisition

For all three subgroups (control - LPFC -RPFC), in the training and test (Retrieval) stages, 10 repetitions of CS alone were used to acquisition the fear extinction memory, only LPFC and RPFC subgroups received electrical stimulation before the start of acquisition. Then the obtained information was evaluated and compared using the Repeated Measure test and SPSS statistical software. After examining the assumption of sphericity (Muchly Test F=0.337) and confirming it, it was found that there is a significant difference $F(2,27)=7.462$, $P=0.003$between the Control group and LPFC and RPFC subgroups (Figure1-Table1). Post Hoc tests were used to determine the statistical difference exists between the Control subgroup and other subgroups. It was found that both HSD and Scheffe post hoc tests showed that there is no significant difference between the control subgroup and the other two subgroups, but there is a significant difference between the LPFC and RPFC subgroups (Fig. 1; Table 1).

The effect of anodal tDCS with TD=18 in the LPFC and RPFC areas on the amount of time latency in fear memory activation

By examining the start time of the freezing in each of the groups in the test phase, the effect of tDCS and the type of protocol and the stimulated region on the time delay in the activation of the fear memory was investigated. One-way ANOVA was used for statistical evaluation. It was shown that there is a significant difference between these three groups. This statistical test showed that there is a significant difference between these three groups. For a more detailed investigation, Tukey's and Sheffy's post hoc tests were performed, which revealed that there is no significant difference between the control

subgroup and the RPFC subgroup, while a significant difference is observed between the control group and the RPFC with the LPFC subgroup (Fig. 2; Table 2).

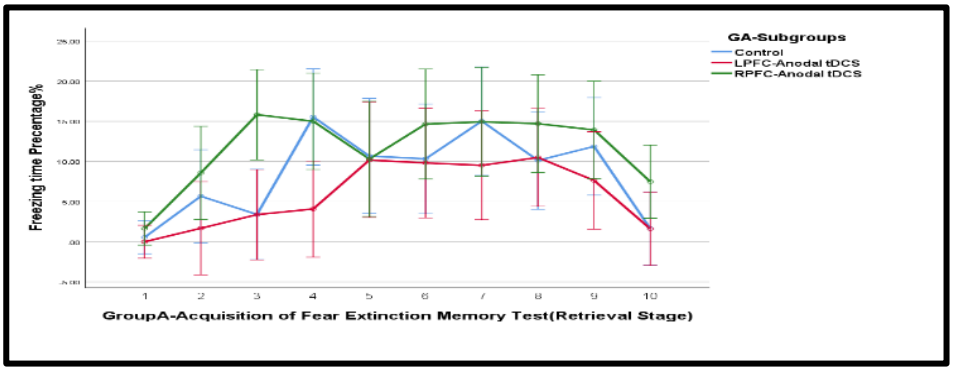


Fig. 1 Group A: Fear Extinction Memory Test Phase (Retrieval Stage)

Table 1. Group A: Repeated Measure Statistical Analysis. Anodal tDCS Fear Extinction Memory.

Between Groups	F(2,27)=7.462	OP=0.915	P=0.003
HSD	Control	LPFC	P=0.21
		RPFC	P=0.105
	LPFC	RPFC	P=0.002
Scheffe	Control	LPFC	P=0.23
		RPFC	P=0.125
	LPFC	RPFC	P=0.003

A (10x10) Experiment: There is a significant difference F(2,27)=7.462 , P=0.003between the Control group and LPFC and RPFC subgroups (Figure 1; Table 1). Post Hoc tests were used to determine the statistical difference exists between the Control subgroup and other subgroups. It was found that both HSD and Scheffe post hoc tests showed that there is no significant difference between the control subgroup and the other two subgroups, but there is a significant difference between the LPFC and RPFC subgroups. It was shown that there is a significant difference between these three groups. This statistical test showed that there is a significant difference between these three groups

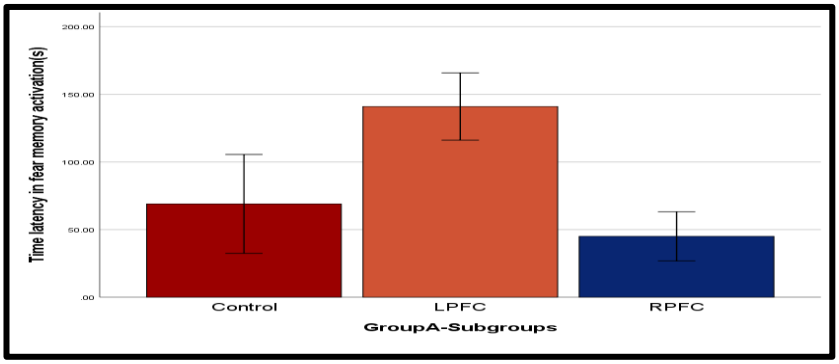


Fig. 2 Group A: Time Latency Test Phase (Retrieval Stage)

Table 2. Group A: One-way ANOVA Statistical Analysis. Anodal tDCS Time Latency

Between Groups	F(2,27)=16.752		P=0.000
HSD	Control	LPFC RPFC	P=0.001 P=0.360
	LPFC	RPFC	P>0.05
Scheffe	Control	LPFC RPFC	P=0.001 P=0.393
	LPFC	RPFC	P<0.05

A (10x10) Experiment: (Figure 2; Table 2). For a more detailed investigation, Tukey's and Scheffe's post hoc tests were performed, which revealed that there is no significant difference between the control subgroup and the RPFC subgroup, while a significant difference is observed between the control group and the RPFC with the LPFC subgroup.

Group B (25x25)

The effect of anodal tDCS with TD=18 in the LPFC and RPFC regions on the fear extinction memory acquisition

30 mice were randomly and equally divided into three subgroups of control, LPFC and RPFC. In the training and test phase, after fear conditioning, all subgroups were subjected to CS stimulation alone 25 times to acquisition of fear extinction memory. Only the two subgroups of LPFC and RPFC in the training phase and before the start of the learning phase were subjected to anodal tDCS with TD=18. The percentage of freezing time in all subgroups was obtained in the test phase. Data were subjected to the Repeated Measure test using SPSS software after confirming the hypothesis of sphericity (GG: Ɛ=0.488). This test showed a significant difference between the three subgroups studied F (2,27) =18.546 , P=0.000. HSD and Scheffe's post hoc test were used to determine the center of this significant difference, which showed that there is a significant difference between the control subgroup and the LPFC subgroup, but there is no significant difference between the control subgroup and the RPFC subgroup. In this group, like group A, there was a significant difference between LPFC and RPFC subgroups (Fig. 3; Table3).

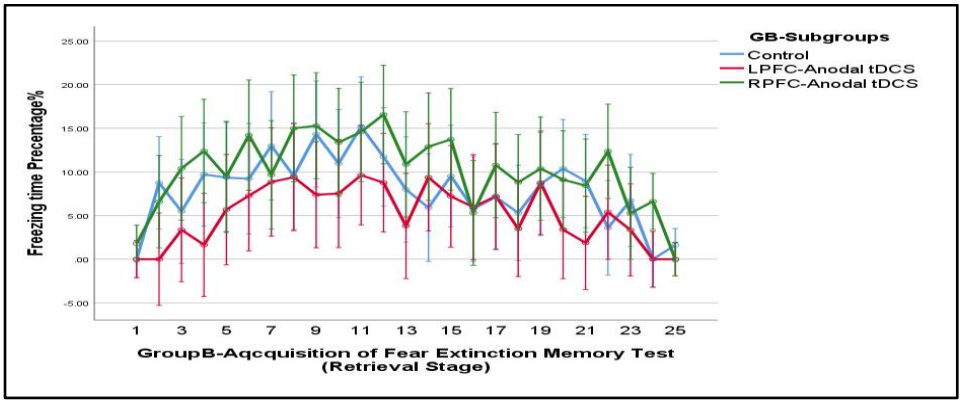


Fig. 3. Group B: Fear Extinction Memory, Test Phase (Retrieval Stage)

Table 3. Group B: Repeated Measure Statistical Analysis. Anodal tDCS Fear Extinction Memory

Between Groups	F(2,27)=15.089	OP=1.000	P=0.000
HSD	Control	LPFC	P=0.013
		RPFC	P=0.056
	LPFC	RPFC	P=0.000
Scheffe	Control	LPFC	P=0.018
		RPFC	P=0.070
	LPFC	RPFC	P=0.000

B (25x25) Experiment: *Figure3-Table3* This test showed a significant difference between the three subgroups studied F (2,27) =18.546, P=0.000. HSD and Scheffe's post hoc test were used to determine the center of this significant difference, which showed that there is a significant difference between the control subgroup and the LPFC subgroup, but there is no significant difference between the control subgroup and the RPFC subgroup. In this group, like group A, there was a significant difference between LPFC and RPFC subgroups.

The effect of anodal tDCS with TD=18 in the LPFC and RPFC areas on the time latency in fear memory activation

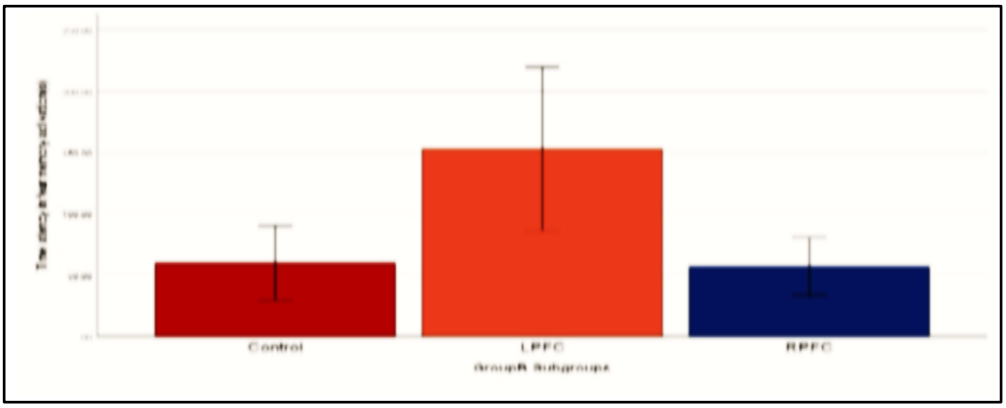
In group B, the amount of delay in the activation of fear memory was evaluated, the results were analyzed by one-way ANOVA in SPSS software. The results of this evaluation showed that anodic electrical stimulation in the LPFC region can delay the onset of fear in the recall stage. In group B, like group A, there was a significant difference between LPFC and RPFC subgroups (Fig. 4; Table 4).

Fig. 4 Group B: Time Latency. Test Phase (Retrieval Stage)

Table 4. Group B: One-way ANOVA Statistical Analysis. Anodal tDCS Time Latency

Between Groups	F(2,27)=7.718		P=0.002
HSD	Control	LPFC RPFC	P=0.007 P=0.994
	LPFC	RPFC	P=0.005
Scheffe	Control	LPFC RPFC	P=0.009 P=0.994
	LPFC	RPFC	P=0.007

B (25x25) Experiment*:* The results of this evaluation showed that anodic electrical stimulation in the LPFC region can delay the onset of fear in the recall stage (Fig. 4; Table 4). In group B, like group A, there was a significant difference between LPFC and RPFC subgroups.



Group C (50x50)

The effect of anodal tDCS with TD=18 in the LPFC and RPFC regions on the fear extinction memory acquisition

30 mice were randomly and equally divided among three subgroups: Control, LPFC and RPFC. All three groups, like the previous two groups, were subjected to the fear conditioning protocol and after confirming the fear conditioning, both in the fear extinction conditioning test phase and in the fear extinction memory train and test phase they were affected by CS alone 50 times. The LPFC and RPFC subgroups were subjected to tDCS before the implementation of the acquisition of fear extinction memory protocol. The results were recorded as the percentage of freezing and were analyzed using the Repeated Measure statistical analysis in spss software after confirming the sphericity assumption of the test. The obtained results showed that there is a significant difference between the control group and the other two groups F (2,27) =15.089, P=0. 000.Tukey's post hoc test showed that there was a significant difference between all three groups. But the other test, Shafi's, showed a significant difference only between the control group and the LPFC group, as well as between the LPFC and RPFC groups (Fig. 5; Table 5).

The effect of anodal tDCS with TD=18 in the LPFC and RPFC areas on the time latency in fear memory activation

In the group, the amount of delay in the activation of the fear memory after creating the fear memory and applying tDCS was investigated in the Control, LPFC and RPFC subgroups. The obtained time data was analyzed using SPSS software and using one-way analysis of variance test, which showed that

there is no significant difference between the control group and RPFC, but there is a significant difference between the control subgroup and the LPFC subgroup (Fig. 6; Table 6).

Fig. 5 Group C: Fear Extinction Memory.Test Phase (Retrieval Stage)

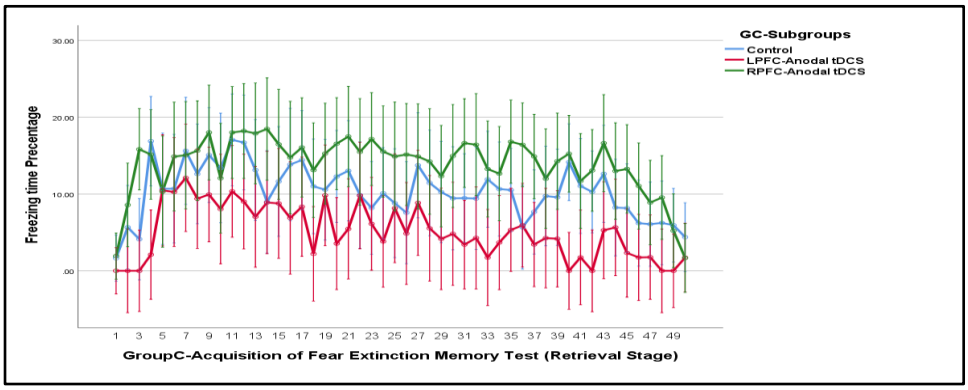


Table 5. Group C: Repeated Measure Statistical Analysis. Anodal tDCS Fear Extinction Memory

Between Groups	F(2,27)=18.546	OP=1.000	P=0.000
HSD	Control	LPFC	P=0.004
		RPFC	P=0.050
	LPFC	RPFC	P=0.000
Scheffe	Control	LPFC	P=0.005
		RPFC	P=0.063
	LPFC	RPFC	P=0.000

C (50x50) Experiment*:* Fig. 5; Table 5: The obtained results showed that there is a significant difference between the control group and the other two groups F (2,27) =15.089, P=0.000Tukey's post hoc test showed that there was a significant difference between all three groups. But the other test, Scheffe's, showed a significant difference only between the control group and the LPFC group, as well as between the LPFC and RPFC groups.

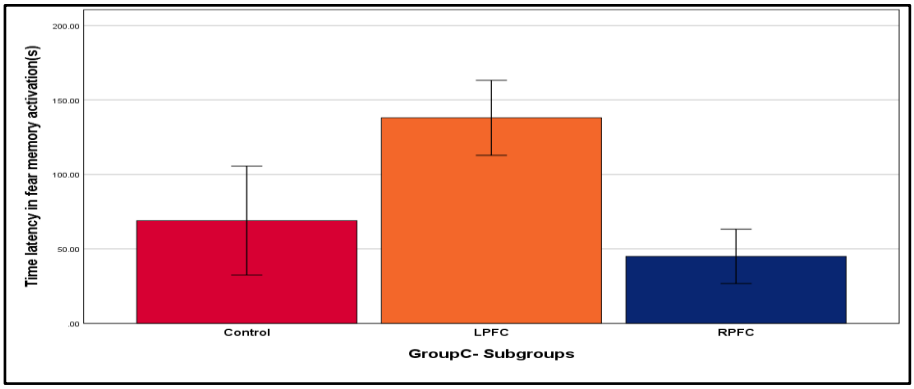


Fig.6 Group C: Time Latency. Test Phase (Retrieval Stage)

Table 6. Group C: One-way ANOVA Statistical Analysis. Anodal tDCS Time Latency

Between Groups	F(2,27)=15.540		P=0.000
HSD	Control	LPFC	P=0.001
		RPFC	P=0.362
	LPFC	RPFC	P=0.000
Scheffe	Control	LPFC	P=0.002
		RPFC	P=0.396
	LPFC	RPFC	P=0.000

C (50x50) Experiment: Fig. 6; Table 6: The obtained time data was analyzed using SPSS software and using one-way analysis of variance test, which showed that there is no significant difference between the control group and RPFC, but there is a significant difference between the control subgroup and the LPFC subgroup.

4. Discussion

Examining the factors that can affect the different stages of fear extinction memory formation is directly or indirectly related to the control and treatment of disorders known as PTSD. Regions of brains such as dACC, Insular Cortex, Hippocampus, Amygdala, vmPC are directly related to the formation of fear memory and fear extinction memory [17, 28-33]. Among the said regions, the PL and IL regions in the mice brain, which are respectively equal to the dACC and vmPC sections in the human brain, and the CE, ITC and B nuclei of the amygdala region, play a very important role in the formation of conditioned fear memory and conditioned fear extinction memory [34-39]. This means that any factor that affects the formation of the conditioned memory of fear or the memory of extinction fear must directly or indirectly affect the activity of these parts. Considering that the formation of fear memory and fear extinction memory through conditioning is the subject of interest in this research, various factors that affect this conditioning must have an effect on the plasticity process in these regions [40]. In addition to the role of different brain regions mentioned in the formation of fear memory and fear extinction memory, the laterality of the brain in humans and rodents(Lateralization) and the difference in the function of the brain hemispheres can also affect the formation of fear memory and fear extinction memory [41, 42]. One of the methods that can be used to influence the said brain regions and brain hemispheres is electrical stimulation of the brain with constant current. Electrical stimulation of the brain with constant and weak currents (tDCS) is one of the most common methods of non-invasive electrical stimulation of the brain. It is important to mention here that such methods do not directly stimulate the neurons of the nervous system, but their mechanism of action is through changing the threshold of stimulation of neurons [43-47]. Anodic stimulation increases the excitability of neurons by reducing the polarization of the neuron membrane and causes an excitatory effect, while cathodic stimulation has an inhibitory effect by causing hyperpolarization in the neuron membrane [43-50]. But it should be noted that the excitatory and inhibitory effect of electrode polarization follows more complex mechanisms, so that the excitatory or inhibitory effect of electrical stimulation cannot always be related to the type of electrode polarization [51-53].

Investigating the effect of anodic electrical stimulation with constant current in the acquisition phase of fear extinction memory

Three subgroups of control, LPFC and RPFC were compared with each other in all the protocols used. In all protocols, all three sub-groups were first subjected to the protocol of creating conditioned fear memory. (For an electric shock with a current intensity of 0.6 mA, 2 seconds). Then only two LPFC and RPFC groups were subjected to anodic tDCS TD=18, and then the training and testing protocol was implemented on all subgroups. Except for group A that a significant difference was obtained only between the LPFC and RPFC subgroups (due to the lack of significant difference between the two subgroups with the control subgroup in group A, this difference is not very reliable), in the rest of the groups a significant difference was observed between the three subgroups of control, LPFC and RPFC. This significant difference can be seen as the result of the application of anodic electrical stimulation in different brain regions, because the protocols for creating fear extinction memory in all three groups were applied similarly for all three subgroups. Based on the results obtained in two groups B and C, it can be seen that anodic electrical stimulation with constant current in the LPFC region has strengthened the formation of fear extinction memory compared to the control but anodic electrical stimulation with a constant current in the RPFC region significantly compared to the control group has weakened the formation of fear extinction memory. Such results may be the result of using TD=18 stimulation and the difference in the performance of the two hemispheres of the brain (Lateralization).

Examining the effect of the type of protocol on the formation of fear extinction memory

If we compare the results of anode electrical stimulation in each area between the three groups, we will notice an important point it can be concluded that with the increase of repetition in each protocol, the statistical difference between the LPFC and RPFC subgroups and the control subgroup is stronger and the degree of significance is higher. It can be seen that in group A, there is no significant difference between the control subgroup and LPFC and RPFC subgroups in both post hoc tests, in group B, there is a significant difference between the LPFC subgroup and the control with both post hoc tests, but no

significant difference is observed between the control subgroup and the RPFC with the two post hoc tests and in group C, there is a significant difference between the control subgroup and the LPFC and RPFC subgroups with HSD post hoc test but with Scheffe post hoc test only between control subgroup and LPFC subgroup significant difference is observed Considering that the type and conditions of electrical stimulation and stimulated areas were similar between the control subgroup and the other two subgroups, it can be concluded that these observed differences between the three groups are the result of using different protocols in the training and testing phases .

Examining the time delay in the activation of fear memory

By examining the time delay of the occurrence of fear in the test (recall) phase of the formation of the fear extinction memory (the time it takes for the mice to show fear-based behavior) in all three groups A, B and C, it was found that there is a significant difference between the control subgroup and the LPFC and RPFC subgroups in all groups. With the post-hoc tests performed in this research, it was found that in all three groups, anodic electrical stimulation using tDCS in the LPFC region delayed the activation time of the fear memory in the test phase, and conversely, such stimulation in the region the RPFC reduced the activation time of the fear memory [54]. In the analysis of the possible cause of such results, it can be argued that the obtained results were compared to the control subgroups, so the creation of fear extinction memory alone cannot be the reason for observing such results but the main cause should be sought in the anodic electrical stimulation and the stimulated regions. In all groups, LPFC and PFC subgroups received the same electrical stimulation and followed the same fear extinction memory protocol, so the only major difference was the regions stimulated. It can be concluded that what these results show is most likely related to the type of brain region that is electrically stimulated. These results should be taken into consideration from a clinical view, and if future research shows such results, it should be confirmed in the human phase in order to use these results in clinical conditions.

5. Conclusion

The results obtained in this research showed that anodic electrical stimulation using tDCS in the LPFC region strengthens the acquisition of fear extinction memory and such stimulation in the RPFC region weakens the acquisition of fear extinction memory. Also, the results show that the increase in repetition in the training phase of fear extinction memory can significantly affect the formation of fear extinction memory and finally, the anodic electrical stimulation of the LPFC in each of the groups was able to delay the activation of the fear memory, and the anodic stimulation of the RPFC accelerated the activation of the fear memory.

Suggestions

Considering that the obtained results are clinically important in relation to PTSD disorders, the following suggestions are presented: 1- The research can be done in the animal phase with a larger number and higher evolutionary level and clinical verification of the obtained results, the human phase of the research is necessary. 2- Stimulation with tDCS in different TDs is recommended in next researches. 3- It is recommended to change the floor and walls of the chamber during the training and test phase to remove the effect of the contexts. 4- It is suggested to use larger chambers in the training and testing phase. 5- If the electronic systems used do not have their own error system, it is recommended to do troubleshooting for them. 6-Finally, it is recommended that the next research be conducted in a multi-effect manner.

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