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Investigation of the effects of direct current stimulation on Parkinson disease in vitro

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Abstract

Aim: Parkinson's disease (PD) is a progressive, neurodegenerative disease characterized by the loss of dopaminergic neurons. Multiple possible mechanisms such as oxidative stress, mitochondrial dysfunction or excitotoxicity caused by glutamate are thought to mediate neuronal loss in PD. It is stated that transcranial direct current stimulation (tDCS) has positive effects on PD, but underlying mechanisms are still largely undefined. So, in this study, the effects of tDCS on PD and the relationship of these effects with glutamate and NMDA levels were investigated.

Materials and Methods: To induce the PD model, 6-OHDA (200 µM) was administered to SH-SY5Y cells for 24 hours. Electrical stimulation was applied to the SH-SY5Y cells at 20 minutes and 7 hours after 24 hours. The effect of tDCS on cell viability was measured by MTT 3-(4, 5-Dimethylthiazol-2-yl) method. Glutamate and NMDA receptor levels were measured using commercial kit.

Results: 6-OHDA increases cell death in SH-SY5Y cells, while electrical stimulation reverses this effect. While 6-OHDA increased the glutamate level, tDCS therapy reversed this effect. There is no significant difference between the groups in NMDA levels.

Conclusion: Our findings suggest that tDCS can be a functional therapy on PD by reducing glutamate toxicity.

Introduction

Parkinson's disease is a progressive neurodegenerative disease with presence of Lewy bodies and dopaminergic neurons degeneration [1]. It is a multi-system neurodegenerative disease affecting the quality of patient's life. Although the basic molecular mechanisms leading to PD are not fully understood, many factors such as abnormal deposition of protein in dopaminergic neurons, mitochondrial dysfunction, oxidative stress, microglial activation and inflammation, cytotoxicity, apoptosis, environmental and genetic factors are associated with disease [2, 3]. Glutamate is one of the most abundant excitatory neurotransmitters in the brain, and impaired neurotransmission in the basal ganglia affects the glutamatergic system [4, 5]. Glutamate released from the presynaptic terminal acts by binding to its receptors on the postsynaptic membrane. Glutamate receptors can be divided into two groups. These are ionotropic glutamate receptors (iGluR) in the form of ion channels and metabotropic glutamate receptors (mGluR) that provide signal transduction via G-proteins. There are three different iGluRs: α-amino-3-hydroxy-5-methyl-isoxazol-4-propionic acid (AMPA), kainic acid (KA), and N-methyl D-aspartate (NMDA) receptors [4]. NMDA receptors, which are heterotetramers, consist of three subunits, NR1, NR2, and NR3 [6]. The NR1 subunit plays a vital role in receptor function by binding the glycine co-agonist. Ionotropic glutamate receptors are ligand-gated ion channels and promote rapid neurotransmission. These receptors affect various excitatory stimuli throughout the central nervous system and are important for various brain functions [5]. With glutamate binding to AMPA or KA receptors, ionotropic channels open and depolarization begins with Na^{+} entry in the postsynaptic neuron. With the effect of depolarization, Mg^{+2} blockade at NMDA receptors is removed and Ca^{+2} entry into the cell is observed through these receptors [7]. Under pathological conditions, glutamate release from the presynaptic membrane can be

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increased or glutamate reuptake function may be impaired [8]. At abnormally high concentrations, it can severely damage neurons through over-activation of NMDA and even lead to neuronal death [9]. There is abnormal glutamate release in the basal ganglia region in PD. This condition has generally been regarded as the result of decreased dopamine levels. It is known that direct or indirect neurotransmitter changes in nigrostriatal pathways affect glutamatergic hyperactivity in Parkinson's disease. Studies show that glutamate-induced excitotoxicity may be the primary cause of dopaminergic neuronal loss, and therefore it is thought that abnormal regulation may contribute to neurodegeneration [10].

The primary medical therapy of PD is pharmacotherapy, including levodopa, but long-term drug therapy has side effects [11]. Therefore, alternative techniques such as deep brain stimulation (DBS) have been used to treat PD and explained to be efficient in improving motor and non-motor dysfunctions [12]. Despite all of them, the high contamination risk and cost relevant to invasive procedures remains a major challenge to be resolved. Furthermore, invasive neurosurgery is applicable if Parkinson's patients meet very specific criteria [13]. In recent years, non-invasive brain stimulation techniques have been investigated as more safer alternatives to modulate cortical excitability [12]. The tDCS, it is a comparatively easy and safety alternative to stimulate cortical excitability by performing a low-intensity current to the scalp [14]. tDCS effects through activation of Na^{+}-Ca^{+2} dependent ion channels and long-term potentiation or depression-like changes in NMDA receptor activity [15]. tDCS protocol, which can ameliorate PD symptoms, would be an important alternative for the treatment.

Human dopaminergic neurons which cells are primarily affected in PD, are quite difficult to obtain and sustain as primary cells, so current PD research is generally preferred with permanently developed neuronal cell models [16]. The SH-SY5Y neuroblastoma cell line is one of the most widely used cell models in PD research because of demonstrating many characteristics of dopaminergic neurons [16, 17]. Furthermore, the SH-SY5Y cell line is demonstrated to express tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, as well as the dopamine transporters [18]. It is thought that SH-SY5Y cells mimic the cellular PD's environment because of all these features we mentioned.

Although studies have revealed the effect of tDCS on the improvement of PD symptoms, the mechanism of this effect has been still not fully elucidated. So in our study, the cellular effects of tDCS and the relationship of these effects with glutamate and NMDA levels were investigated in vitro PD model.

Materials and Methods

Cell culture

SH-SY5Y cells were received from Medical Pharmacology Department at Ataturk University (Erzurum, Turkey). The SH-SY5Y cell line was cultured in DMEM with 10% FBS and antibiotic solution. After the cells were incubated at 37 °C with 5% CO_2, 0.3 × 10^6 SH-SY5Y cells were seeded in 6-well plates. 6-OHDA (200 µM) was applied to each well for one day (24 hours) to mimic PD in the cell line. Electrical stimulation was applied to the SH-SY5Y cells at 20 minutes and 7 hours after 24 hours. When the procedure is finished, the MTT test and kit procedures were applied [19, 20].

MTT analysis

MTT method was used to evaluate cell viability. 20 µl of MTT solution (Sigma-Aldrich) was added to each well. After four hours, supernatants were collected and 150 µm of DMSO was added, then the absorbance was measured at 490 nm [20].

Establishment of electrical stimulation for cell culture

A standard 6-well cell culture plate was used to generate Electrical Stimulation (Estim). 6-well plate cover was drilled each of the six wells (12 holes in total). Platinum wires were cut and bent into L-shape (3 cm) then inserted into the holes. Platinum wires were fixed to the cover holes with adhesive and left to dry. Six platinum wires coming out of the caps served as cathodes, and the remaining six platinum wires served as anodes. When the lid was closed, the platinum wires (anode and cathode tips) were mounted without touching the cells in the subfloor culture plates.

The voltage (2.5V) and current of Estim, which are transmitted to the cells by arranging a DC power supply, are adjusted [21, 22]. It was shown in Figure 1.

Glutamate and NMDA levels

Glutamate and NMDA determination in cells was measured using the Bioassay Technology Laboratory (BT-Lab, Shanghai, China) (Cat. No. E4078Hu, Cat. No. E1051Hu) ELISA Kit respectively according to manufacturer's instructions [23].

Statistical analysis

All analyzes were performed using Statistical Package for the Social Scineces (SPSS v.22, SPSS Inc., Chicago, IL, USA) and shown as mean±SD. To assign normality and homogeneity, Shapiro-Wilk and Levene tests were employed. The Kruskal-Wallis test was used for the parameters that did not show normal distribution. Results were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test in normal distribution ($p<0.05$).

Results

MTT

6-OHDA caused significantly decrement in cell viability ratio in the MTT assay. Cell viability was accepted as 100% to control group and given as %. 6-OHDA group (63.56±0.13, $p<0.05$) had significantly less cellular viability ratio than the control group. Cell viability ratio at 6-OHDA_20 min is 64.85±0 and at 6-OHDA_7h is 44.74±0.66. tDCS treatment at 20 min promoted a significant increase in cells as compared with 6-OHDA ($p<0.05$) but decrease at 7h ($p<0.05$). The increment at the 20th minute showed that the neurotoxic effect of 6-OHDA on

Table 1. Glutamate and NMDA Levels Use dot mark in stead of comma.

	C	C_20min	C_7h	6-OHDA	6-OHDA_20min	6-OHDA_7h
Glutamat	20.98±0.86	20.61±0.33	20.97±1.43	24.00±0.40 *	23.18± 0.47	21.03±1.04 #
NMDA	25.72±0.94	23.77±0.62	21.65±1.08	24.28±1.48	27.36± 1.08	24.93±2.99

* p < 0.05 difference from C group, # p < 0.05 difference from 6-OHDA group.

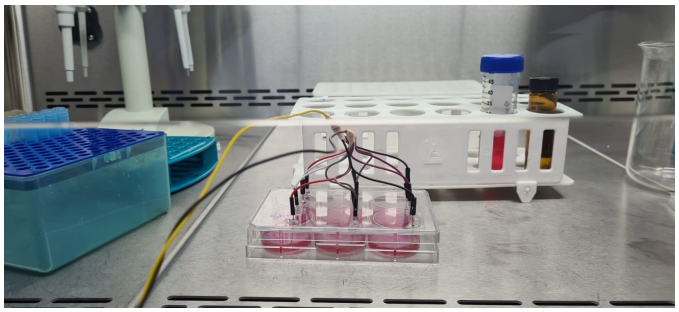

Figure 1. Electrical Stimulation Setup.

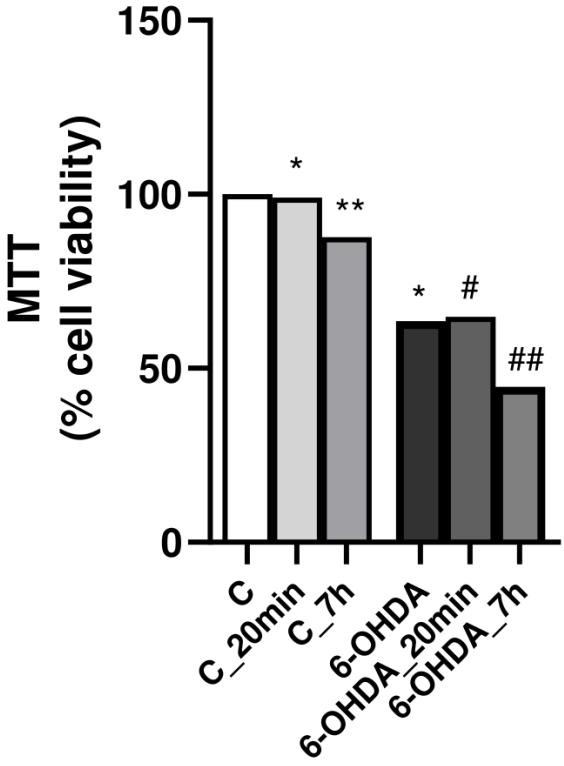

Figure 2. Effects of tDAS on cell viability (MTT) * p < 0.05, ** p < 0.01 difference from group C, # p < 0.05, ## p < 0.01 difference from 6-OHDA group.

neuronal cells had been reversible with tDCS and significantly increased the rate of viable cells. However, late treatment was insufficient to have an effect because cell death was high at the 7th hour. (Figure 2).

Glutamate and NMDA level

No change in time-dependent glutamate levels was shown in the control groups. Nevertheless glutamate levels increased significantly in the 6-OHDA according to the control group ($p<0.05$). A decrement was observed in the 6-OHDA_20m group compared to the 6-OHDA group. Glutamate level was significantly lower in the 6-OHDA_7h group according to the 6-OHDA ($p<0.05$) (Figure 3A, Table 1). There was no statistically significant change in NMDA levels between groups and depending on time (Figure 3B, Table 1).

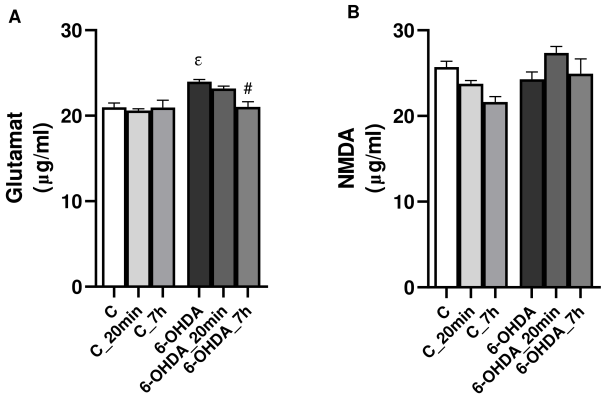

Figure 3. A) Glutamate Levels, B) NMDA Levels. * p < 0.05 difference from C group, # p < 0.05 difference from 6-OHDA group.

Discussion

PD is mainly identified by the loss of dopamine neurons, but further elucidation of the mechanisms leading to neuronal death and possible treatment modalities are needed. Studies show that the glutamatergic signal plays a pivotal role in the pathogenesis of the disease [5, 24]. As an major excitatory neurotransmitter, glutamate is closely associated with the emergence and development of PD [5, 10]. In 2013, the study by Ballaz et al. proved that SH-SY5Y cells were exposed to glutamate toxicity and ascorbate, nonenzymatic antioxidant, could prevent cell death [25]. Glutamate caused dose-dependent toxicity in dopaminergic cells, mainly via stimulation of AMPA and metabotropic receptors and to a lesser extent NMDA and kainate receptors. In our study, it was shown that cell viability decreased in the 6-OHDA group compared to the control group, the application of current at the 20th minute tended to provide a protective effect by increasing cell viability. While no time-dependent change was observed in the glutamate level in the control group, a significant increase was found in the glutamate level in the 6-OHDA group and a decrease in glutamate levels was detected in 6-OHDA_7h. Therefore, current application may mediate neuronal protection by reducing glutamate-mediated neurotoxicity. Our findings

demonstrate the importance of current application as neuroprotective and highlight its role in glutamate excitotoxicity. Our findings support the hypothesis in the literature that glutamate dose-dependently induces degeneration of dopaminergic cells, and this transmitter may cause excitotoxicity in human dopamine cells [26, 27]. NMDARs have the high affinity for glutamate and are highly regulated by the central nervous system. NMDARs activation can promote neuronal vitality via Ca^{2+}-mediated signal transduction, however over excitation can promote neuronal death [28, 29]. High extracellular glutamate levels cause to overstimulation of Ca^{2+}-permeable NMDARs, followed by increased Ca^{2+} level and excitotoxicity [10]. In our study, we observed that 6-OHDA increased the glutamate level, while tDCS application reversed this effect. NMDA receptors have been shown to be hypersensitive to endogenous glutamates in the dopamine-depleting striatum [30]. An increased response to glutamate can reflect changes in the functioning of the receptor channel, such as increased channel opening frequency or longer duration of opening. Physiologically, this response can cause greater Ca^{2+} influx to the post-synaptic neuron via the NMDAR. Numerous studies have reported alteration in NMDAR subunit phosphorylation in PD model [30, 31]. The data collectively show increased tyrosine phosphorylation of lesioned NR2B subunits according to non-lesional striata [31, 32]. Tyrosine phosphorylation of NR2 subunits affects NMDA function and thus Ca^{2+} flux at the receptor. It is probable that the observed increased response of striatal NMDA receptors to glutamate is due to altered tyrosine phosphorylation of NMDA receptors. There are arguments suggesting that NR1 subunit levels are not changed in the lesioned striata of 6-OHDA-Lesioned rats [30]. Total NMDA levels did not change between groups in our study statistically. It has been well known that NMDA receptor activation is increased in PD, but this increased ability of glutamate cannot be associated with the increased number of NMDA receptors. There are numerous ways to modulate NMDA receptors. These include distinct subunits, transport of receptors to synaptic sites, phosphorylation of NMDA subunits and binding of receptors to secondary messenger systems. Considering the studies emphasizing that only NR1 subunits of NMDA receptors are expressed in SH-SY5Y cells, our findings are consistent with the literature [33].

tDCS is one of the non-invasive brain stimulation methods [34]. Despite numerous preclinical and clinical studies, the main mechanism of action of tDCS has not been fully elucidated yet. Although some potential mechanisms have been suggested, they are still insufficient to explain the whole picture. tDAS application mainly increases electrical activity and cortical excitability around the anodal electrode [35, 36]. Recent studies show that tDCS, together with rehabilitation, has long-term effects in improving symptoms in various neurological disorders [36]. Our results show that tDCS application can reduce excitotoxicity by decreasing glutamate level and may be one of the promising treatment methods for PD.

The SH-SY5Y cells is a basic and inexpensive in vitro experimental model to study pathophysiological mechanisms of PD and developing new pharmacological treatments [37, 38]. In our study, cell viability was measured with the MTT test. We observed that 6-OHDA reduced cell viability in SHSY5Y cells and tDCS treatment at 20 minutes increased the viability of the cells. To the best of our knowledge, this is the first study to investigate the effect of tDCS on glutamat and NMDA levels in PD.

In future studies, it is planned to investigate the effects of tDCS on the phosphorylation of NMDA receptors and excitatory amino acid transporters that have a major role of glutamate uptake from the synaptic cleft. We also plan to evaluate the neuroprotective effects of tDAS against oxidative damage and neuroinflammation in the 6-OHDA-induced PD model.

Conclusion

Overall, these findings showed that tDCS may contribute to the recovery process of Parkinson's disease by reducing glutamate toxicity. tDCS treatment increased the viability of the cells, so tDCS could be a functional alternative therapy on PD, tDCS may contribute to the recovery process of Parkinson's disease by reducing glutamate toxicity.

Ethical approval

It is a study that does not require an ethics committee.

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