The Effects of Transcranial Electric Stimulation and Cognition Reinforcement Training on the Expression of Tau Protein in Alzheimer’s Disease Rat Models

This study is intended to examine the tDCS and Morris Water maze training in Alzheimer’s disease (AD) rats on Tau protein expression. Experiment groups were divided into four groups and assigned 16 rats to each group. Group I was a control group (AD induced by scopolamine); Group II was a experimental control group (AD injured by scopolamine and treatment tacrine); Group III was a group of tDCS application after AD injured by scopolamine; Group IV was a group of morris water maze training after AD injured by scopolamine. In cognition test, the outcome of group II was significantly lower than the group I (p<.001), and group III, IV were significantly low result at 14 days (p<.05). In histological finding, the experimental groups were destroy of micro vessels and finding of cell atrophy and swelling. Group III, IV were decreased in degeneration of liver and kidney cells. In immune—histochemistic response of BDNF and tau protein in hippocampus, BDNF expression of Group II was more increase than the other groups, and increase of BDNF expression was III, IV were higher than group I at 21 days. Tau protein expression of Group II was more decrease than the other groups, and decrease of Tau protein expression was III, IV were lower than group I at 21 days. These result suggest that improved tDCS and morris water maze training after scopolamine induced is associated with dynamically altered expression of BDNF and Tau protein in hippocampus and that is related with cognitive function.

Keywords: Alzheimer’s Disease; tDCS; Morris Water Maze Training; Tau

INTRODUCTION

Alzheimer’s disease (AD) is the most common neurodegenerative dementia among senile disorders causing complex cognitive functional deficiency including memory disorders to the extent that the activities of daily living are seriously interrupted(1). Cognitive functions are the ability to understand and learn things happening in daily living environments and a series of composite thinking processes of learning through experience and making decisions(2). Cognitive functions integrate auditory, linguistic, and visual senses as well as perception as fundamental areas and include higher level functions such as attention, memory, planning ability, organizing skills, problems-solving skills, and abstracting ability(3). It has been reported that when cognitive functional disorders occurred, memory and concentration declined, disorders occurred in the perception of spaces, and visual and perceptive abilities decreased(4). Learning undergoes recognition and association levels to reach an automation level. Learning is a process of drawing continuous behavioral changes occurring as a result of past experience and practice. Neurons proliferate and the brain is reorganized as a result of learning. Cognitive functions are necessary for such learning. Cognitive functions are constructed based on memory ability. Intervention processes using physical therapy implemented through task-oriented cognitive function reinforcing training are necessary to enhance cognitive functions. Recently, therapeutic concepts in the aspect of cognition are dealt importantly in the field of physical therapy.

Direct cerebral electric stimulation is used to reinforce cognitive functions. This transcranial electric stimulation has been known to provide direct non-invasive stimulation to human brains thereby
promoting the reversibility of the brain. The transcranial electric stimulation is a method of selectively changing the excitability of local regions of the brain by applying currents of 1–2 mA(5). This method can selectively activate the brain by adjusting the polarity, stimulating periods of time, and intensity of the stimulating current(6, 7). The physiological effects of transcranial electric stimulation are made by reversible changes in the synaptic connectivity that shows changes in the efficiency of NMDA(N-methyl-D-aspartic acid) receptors in the nervous system(8).

It has been reported that the anode locally reduces GABA which is an inhibitory neurotransmitter and the cathode suppresses glutamatergic nervous activities(9). Therefore, the present study is intended to examine the effects of non-invasive electric stimulation on the activity of the brain and the resultant enhancement of cognitive functions using transcranial electric stimulation.

Motions cannot be separated from recognition and perception since they are composed of independent components from recognition and perception(10). Motions are learned through repeated experience and training and neurons proliferate and the brain is reorganized as a result of learning(11). Motions are positively involved in the protection and regeneration of nerves damaged in central nervous system(CNS) diseases and enhance or maintain functional movements, flexibility, muscle strength and endurance, and walking speeds(12). The most frequently used method in previous studies that examined the effects of motions on cognitive ability using experimental models is considered to be important basic scientific data for cognition related recovery.

METHODS

Experimental Animal

In the present study, AD was induced to 100 Sprague–Dawley white rats(8 weeks old, male, Damul Science) weighed 230±20g using Scopolamine of them, 64 rats that showed cognitive function disorders were randomly assigned to four groups of 16 rats for the experiment. Temperatures in the feeding room were set to 22±1°C and humidity was maintained at 55±10 %. The contrast cycle was set to 12 hours. These conditions of the feeding room were constantly maintained for the term of experiment. Solid feed(Samyang Co., Ltd., Korea) was used diets and the animals were allowed to freely take water and feed.

Biological behavioral responses related to the recovery of cognitive functions were evaluated immediately after inducing cognitive function disorders and 7, 14, and 21 days later. Immunohistochemical examinations were conducted with samples made on the 21st day sacrificing 8 animals per group.

Experimental Method

Induction of cognitive function disorders using scopolamine

Scopolamine(Sigma, S1875, USA) 1mg/kg was dissolved and diluted in 0.9 % saline solution and intraperitoneally injected into normal white rats for 30 days(once a day) to induce cognitive function disorders. To compare with the Scopolamine induced experimental group, tacrine(Sigma, A3773, St. Lous, MO, USA) 10mg/kg that detoxifies the animals was dissolved and diluted in 0.9% saline solution and intraperitoneally injected for 4 weeks(once a day) into the experimental control group that had been administered with Scopolamine(Table 1).

<table>
<thead>
<tr>
<th>Table 1. Classification of experimental groups</th>
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<tr>
<td>Groups</td>
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<tr>
<td>I(n=16)</td>
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<td>II(n=16)</td>
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<td>III(n=16)</td>
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<td>IV(n=16)</td>
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Transcranial direct current stimulation (tDCS)

In the present experiment, the tDCS was applied using direct current stimulators (Cyber medic Co., Jeonju, Korea) that can be adjusted in intensity by 0.1 mA at a time in accordance (20). To attach anode electrodes to the transcranial regions of the cerebral cortex of the experimental animals, pads were made in a size of 1 cm width × 1 cm length and plastic cups that fit the size of the heads of the experimental animals were made to cover the heads of the experimental animals after attaching the pads to the transcranial regions in order to prevent the pads from moving. Cathode electrodes were applied to the bodies of the animals to prevent electric shunting effects. To reduce electric resistance between the skin and the electrodes, gel was used after shaving. The plastic cups were filled with the gel, put on the heads of the animals, and fixed. The intensity of the electric current applied to the animals was set to 0.1 mA which was a half of the intensity at which all the whiskers of the white rats visibly contracted and the electric current was applied two times per day for 20 minutes, five times per week, for 4 weeks at the same time points every day.

Cognitive enhancing training (Morris water-maze training)

To enhance the animals’ neurologic cognitive functions, underwater training was implemented using a Morris Water-maze. A round water tank 150 cm in diameter and 50 cm in height was filled with 25°C water up to 30 cm and a round escape platform 15 cm in diameter and 28 cm in height was installed in the water tank to apply underwater training to the animals. The inside of the Morris Water-maze was painted in black so that it could be distinguished from the white color of the white rats. An experimental stand, a chair and an experimenter were always placed at the same locations outside the water-maze so that they could be utilized as external clues of the maze and black India ink was dissolved in the water in the water tank so that the round escape platform would not be seen and thus could not be used as a visual cue.

All the white rats used in the experiment were trained two times a day for 180 sec each time for five days before cognition function disorders were induced. After the induction, the Morris water maze training for experimental group IV was set to be implemented until the rats find the round escape platform from the date of beginning of motions and applied two times a day for four weeks.

Result Measurement

Spatial learning and memory ability test

Spatial learning ability related to cognitive functions was tested using Morris water maze test before inducing cognitive function disorders, and 7, 14, and 21 days later. The water maze was divided into the same quadrants: northeast (NE), northwest (NW), southeast (SE), and southwest (SW) quadrants. The escape platform was placed on the center of the southwest quadrant and one of the remaining quadrants was used as a starting position. The evaluation was conducted by measuring and analyzing the lengths of time taken by the white rats in the water tank to go on the escape platform after staring.

Histological assessment

Each of the white rats in each experimental group was anesthetized using 2% enflurane (Isoflurane, Choong Wae Pharmaceutical Corporation, Korea) in oxygen and nitrogen mixed at a ratio of 3:7 at 21 days after the administration of Scopolamine, its thoracic cavity was opened and a cannula was inserted into its ascending aorta through its left ventricle. Thereafter, the rat was perfusion-washed with 0.9% NaCl 200 ml using a perfusion washer and perfusion-prefixed using 4% paraformaldehyde dissolved in 0.1M phosphate buffer saline (PBS) solution. Thereafter, the brain removed of cranial nerves, and the liver and the spleen in the abdominal cavity were extracted and post-fixed by being immersed in 10% paraformaldehyde solution for 24 hours. The post-fixed tissues were automatically treated for 14 hours through processes of dehydration, transparency, and infiltration using an automatic tissue treating machine (4640B, Sakura, Japan). Thereafter paraffin blocks were made with the tissues using an automatic embedding device (Tissue-Tex, Japan). The produced paraffin blocks were sliced into 4 μm thick pieces at intervals of 180 μm from the top of the prefrontal lobe using a Rotary Microtome 2040 (Japan). The slices were put into a flotation water tank and then taken out and attached to gelatin-coated slides. For histological observation, the slices were stained using the normal H&E staining method and made into permanent samples by sealing them with Canada balsam (Sigma, USA). For morphological observation, the slices were photographed using an optical microscope (Olympus Bx 50, Japan) with the CCD camera (Toshiba, Japan) installed in the microscope and structural changes in the cells, etc. were observed.
Immunohistochemical assessment
To examine the expression of BDNF and Tau proteins, immunohistochemical response tests were conducted. After undergoing a deparaffin process, the slides were subjected to microwaving in 30% Tris-EDTA in order to remove non-specific reactions to be prepared for immunohistochemical staining and cooled for 20 minutes. Thereafter, as a pretreatment process, the activity of intrinsic peroxidase was blocked using 3% hydrogen peroxidase. Then, a PBS mixture solution added with 15% blocking serum was used so that the primary antibody solution could be easily absorbed into the tissues. The tissues were washed many times with 0.01M PBS, subjected to reactions with BDNF and Tau proteins' primary antibodies (SantaCruz, USA) diluted in PBS at a ratio of 1:300 at 4°C overnight, washed with PBS, and subjected to reactions with universal antibody for 90 minutes. The tissues were again washed three times for 10 minutes each time using 0.01M PBS, subjected to reactions in streptavidin for 30 minutes at room temperature, washed with PBS, and subjected to color formation for 10 minutes with DAB (3,3'-Diaminobenzidine, 60382248, ZYMED Lab, Germany). The tissues were again washed PBS, contrast-stained with Hematoxyline, washed with flowing water, dehydrated through immersion in 80%, 90%, 100% ethanol for 10 minutes respectively, treated with 100% xylene two times for 10 minutes each time as a process for transparency, and made into permanent samples by sealing with Canada balsam (Sigma, USA).

Data Analysis
In the present study, SPSS ver. 18.0 for Windows was used for statistical analysis and individual resultant values were indicated as means and standard deviations. One-way ANOVAs were conducted for comparison of behavioral measured values of individual groups by measuring time and Tukey’s multiple range tests were conducted for post hoc tests. Repeated measures analysis of variance was conducted to test the significance of the assessment of spatial perception learning ability by applied time. The significance level was set to α = 0.05.

RESULTS
Spatial Learning and Memory Ability Test
The results of measurements of the time taken by the white rats in the water tank to go up the escape platform after starting using Morris water maze learning processes were as follows (Table 2).

With regard to differences between the groups at individual measuring times, significant differences began to occur at day 7. According to the results of post hoc tests, the time taken decreased more significantly in group II compared to group I at day 7 (p < 0.001), the most significantly in group IV (p < 0.05) followed by group III, and group II (p < 0.01) in order of precedence compared to group I at day 14, and the most significantly in group IV (p < 0.01) followed by

<table>
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<th>Groups</th>
<th>Pre (sec)</th>
<th>7 days (sec)</th>
<th>14 days (sec)</th>
<th>21 days (sec)</th>
<th>F-value (day)</th>
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<tr>
<td>I (n=16)</td>
<td>58.5±18.00</td>
<td>57.38±20.22</td>
<td>57.06±21.2</td>
<td>56.38±23.15</td>
<td>495.67***</td>
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<tr>
<td>II (n=16)</td>
<td>59.75±16.46</td>
<td>53.13±9.27†</td>
<td>46.31±7.92†</td>
<td>40.31±7.45 †††</td>
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<tr>
<td>III (n=16)</td>
<td>58.94±13.82</td>
<td>54.13±7.97†</td>
<td>49.44±10.74†</td>
<td>45.94±12.79†</td>
<td></td>
</tr>
<tr>
<td>IV (n=16)</td>
<td>58.50±18.00</td>
<td>53.44±16.21</td>
<td>48.75±16.47†</td>
<td>44.31±14.72†</td>
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<tr>
<td>P-value</td>
<td>.805</td>
<td>.009</td>
<td>.00</td>
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All values showed mean±SD.
Tested by repeated measures ANOVA and there were significant differences among the four groups and days, (**: p < 0.001, †: p < 0.05, ††: p < 0.01, †††: p < 0.001)
Group I : Scopolamine induce + nontreatment  Group II : Scopolamine induce + Tacrine
Group III : Scopolamine induce + tDCS  Group IV : Scopolamine induce + Moriss water maze training
group II (p<.05), and group II (p<.001) in order of precedence compared to group I at day 21. In particular, among differences in individual groups compared to group I at day 21, the statistically significant decrease in group IV was much larger than that in group III.

Repeated measures analyses were conducted to identify changes in resultant value in individual groups among measuring times. Changes among the experimental groups and among measuring times showed significant differences respectively (p<.001).

**Histological Finding**

H & E staining was conducted to identify histological changes in the liver and the spleen appearing due to the toxicity of Scopolamine used to induce AD (Fig. 1, 2).

Whereas experimental group I showed hepatic cell cords well arranged centering on the central vein and well preserved formal liver lobular structures centering on the portal vein, experimental group II administered with Scopolamine showed characteristic findings of vacuolar fatty liver indicating that there was hepatotoxicity resulting from Scopolamine administration. Many large and small vacuoles resulting from fat accumulation were observed in liver cells and findings of nuclei pushed out to surroundings of cells by accumulate fat were observed in some cases. Damage to liver cells similar to that in experimental group I was identified in experimental groups III and IV and thus it could be identified that vacuolar lesions were mainly observed in the regions around the portal vein and that the vacuolar lesions decreased to some extent in the regions around the portal vein indicating some recovery (Fig. 1).

As histological changes in the kidney, the construction of capillary walls around the glomerulus was destroyed and drop-outs of surrounding glomerular capsules were observed in experimental group I. Renal corpuscles were not observed and findings of the nuclei of epithelial cells generally pushed out to the surroundings were observed. However, in experimental group II, glomerular capsules were well surrounded by epithelial cells and substrates were well formed with capillaries, basement membranes and epithelial cell processes. Although spleen tissue cell damage similar to that in experimental group I was identified in experimental groups III and IV, findings of capillary expansion and local thickening and adhesion of basement membranes were observed (Fig. 2).

At day 21, BDNF expression was observed in individual groups with dark brown cell bodies and long cell processes. The lowest degree of expression was observed in experimental group I. However, relatively high degrees of expression were observed in experimental group II. Although higher degrees of expression were observed in experimental groups III and IV compared to experimental group I, very high degrees of BDNF expression could not be observed.

**Fig. 1.** The histological finding on liver
H&E stain, ×200, 21days, a: group I, b: group II, c: group III, d: group IV

**Fig. 2.** The histological finding on kidney
H&E stain, ×200, 21days, a: group I, b: group II, c: group III, d: group IV

**BDNF’s Immunohistochemical Responses in Hippocampus**

At day 21, BDNF expression was observed in individual groups with dark brown cell bodies and long cell processes. The lowest degree of expression was observed in experimental group I. However, relatively high degrees of expression were observed in experimental group II. Although higher degrees of expression were observed in experimental groups III and IV compared to experimental group I, very high degrees of BDNF expression could not be observed.
However, given that BDNF expression was observed in experimental groups III and IV, it could be identified that cytohistological changes occurred in the CA1 region of the hippocampus (Fig. 3).

Hyperphosphorylation of Tau protein reduces the binding ability to connect microtubules with each other and Axons do not grow well in phosphorylated tau protein. At day 21, Tau protein was expressed in Axons and neurons in individual groups and the expression was irregular. The highest degree of expression was observed in experimental group I. Relatively low degrees of expression were observed in experimental group II compared to other groups. Although relatively low degrees of expression were observed in experimental groups III and IV compared to experimental group I, irregular expression of Tau protein was observed. Therefore, decreases in Tau protein expression could be identified in experimental groups III and IV (Fig. 4).

rTMS and tDCS used during rehabilitation training periods are types of intervention necessary to check the recovery of brain functions and can provide unspecific inputs to the cerebral cortex motor system that must promote changes in neuronal activities and Synapse reversibility. Resultant changes in the strength of synapse junctions become to exist first as inevitable stages toward the recovery of motor skills in most cases (21). Many hypotheses are presented as mechanisms for tDCS to affect the activation of the cerebral cortex. According to studies conducted thus far, the mechanisms are considered related to increases in excitatory glutamatergic cortical pathways and intracortical inhibitory pathway suppressing effects (5). However, to determine accurate mechanisms, still more study outcomes are coming to the fore. Liebetanz et al. (5) found that carbamazepine which is a Na+ pathway blocker served the role of stabilizing voltage dependent membrane potential and selectively removed the effect of anode transcranial electric stimulation and reported that the effect of anode transcranial electric stimulation was related to the depolarization of membrane potential.

It has been reported that in the hippocampal dentate gyrus closely related to cognitive functions, new neurons are generated even when the growth of hippocampal dentate gyrus has been completed (22). The generation of neurons in the hippocampal dentate gyrus as the center of learning and memory is enhanced by repeated learning, hormone secretion such as Serotonin and Estrogen, and motor stimulation while being suppressed by stress, aging, adrenal cortex hormones, etc. (23). The CA1 region of the hippocampus which is an important anatomical structure for memory functions responds very sensitively in various kinds of brain damage such as ischemia, hypoxia, and glycopenia. The degeneration
of the CA1 and CA2 regions of the hippocampus was suggested to be closely related to damage to memory in an animal experiment(24) reported that ischemic damage to the hippocampus induced spatial memory impairment. Onodera et al.(25) advised that the loss of neurons and axon damage in the CA1 region of the hippocampus caused prolonged spatial memory deficit. White rats damaged in the region of the hippocampus due to Scopolamine injections show disorders in spatial learning ability in motor behavioral cognitive ability tests such as Ziggurat task and 8 shaped maze tests and show cognition function disorders in related tests such as Morris water maze because the region of the hippocampus is related to learning and memory. Therefore, to implement two therapeutic interventions: cognitive reinforcing training and tDCS application and identify the recovery of cognitive functions, the present study was intended to check the degrees of expression of BDNF and Tau proteins in the CA1 region of the hippocampus with Morris water maze assessment and immunohistochemical changes.

In Morris water maze tests which are space perceptual learning ability tests, significant differences occurred between the groups at day 7 after intervention and thereafter. According to the results of post hoc tests, the time taken decreased more significantly in group II compared to group I at day 7 (p<.001), the most significantly in group IV (p<.05) followed by group III, and group II (p<.001) in order of precedence compared to group I at day 14, and the most significantly in group IV (p<.01) followed by group II (p<.05), and group II (p<.001) in order of precedence compared to group I at day 21. In particular, among differences in individual groups compared to group I at day 21, the statistically significant decrease in group IV was much larger than that in group III. The values obtained in Morris water maze tests at different time points of measurement were maintained for the longest time in experimental group II injected with Tacrine followed by experimental group IV and experimental group III in order of precedence. Given this, it is considered that the number of times of feed eating and errors maintained to some extent even after the intervention because of the recovery of motor skills and the simple nature of the maze. Experimental group I kept showing disorders at different time points of measurement without any change over time while feed obtaining time was shortened in other groups. These results were consistent with the results of previous studies that considered these results as meaning the enhancement of cognitive functions(26). The experimental groups applied with Morris water maze and tDCS respectively are considered to have been improved in spatial learning ability and memory because these were learned through appropriate feedback and willfully repeated practice through willful repetition became learning. The spatial learning ability and memory are considered to have been learned through practices in similar situations based on the principle of similarity which stimulated the hippocampus for nerve generation. Given the changes in experimental group III, not only Morris water maze but also the application of tDCS are considered to have therapeutic effects on cognition function disorders appearing due to AD. This could be also identified with changes in BDNF and Tau proteins.

In the case of BDNF which is a neurotrophic factor appeared in the CA1 of the hippocampus through immunohistochemical analysis, the degree of expression was relatively well maintained in experimental groups II and IV at day 21 after the application of intervention. The expression of Tau protein that is related to AD decreased in experimental groups II, III, and IV compared to experimental group I at day 21 after the application of intervention. In particular, experimental group II showed clear decreases in immunity and this was consistent with the improvement of cognitive functions shown by the decrease in the time to obtain the feed in the Morris Water maze. The amounts of changes in BDNF, Tau proteins in the hippocampus observed at day 21 after intervention meant that the effects on cognitive functions were maintained for a long time after the application of intervention consistently with the results of previous studies indicating that motions had effects on BDNF(22, 27) and Tau proteins(28) related to cognitive functions. When cognitive function disorders are severe after the onset of AD, Ach E suppressants such as donepezil, tacrine, and galantamine are used in clinics to prevent fast decomposition of Ach in order to maintain cognitive functions and memory(29). Although the use of Ach E suppressants as such does not cause serious adverse effects, those drugs commonly cause cholinergic adverse effects such as nausea, anorexia, vomiting, and diarrhea and effective periods are short. Therefore studies of tDCS as a medical technology that can offset the adverse effects of Ach E suppressants and control the long-term tolerance of drugs are considered more necessary. Furthermore, interventions combining cognitive reinforcement training and tDCS are considered to have the highest therapeutic value,
When the aforementioned contents are synthesized, cognitive reinforcement training and tDCS are considered stimulating and promoting neuronal activities in AD induced white rats thereby activating neurotransmitters in the cerebral cortex and the hippocampus. Cognitive reinforcement training and tDCS applied to AD patients with cognitive function disorders are considered to have more positive effects on cognitive functions and maintain cognitive functions for a long time.

CONCLUSION

To examine the effects of tDCS and cognitive reinforcement training on the cognitive functions of AD white rat models, cognition function disorders were induced using Scopolamine and cognition related behavioral responses were assessed and immunohistochemical changes in hippocampus tissues were observed. According to the results, in Morris Water maze, experimental group II showed the most significant effects and experimental groups III and IV showed significant differences. The expression of BDNF and Tau proteins in the CA1 region of the hippocampus was observed in the results of immunohistochemical tests. According to the results, in the case of BDNF, experimental group II showed the most significant expression and experimental groups III and IV also showed significant expression. In the case of Tau protein, the opposite results were observed. Based on the aforementioned results, it was identified that the application of tDCS and cognitive reinforcement training after the induction of cognition function disorders of AD positively affected the recovery of cognitive functions and immunohistochemical changes and that the effects were maintained for a certain period of time. Therefore, only cognitive reinforcement training mainly using body movements had significant effects on cognitive functions too and in particular, tDCS that can change the excitability of brain cells as a non-invasive method could be presented as a new treatment method for patients with cognitive function disorders.

REFERENCES

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