A Study on the Effects of Needle Electrode Electrical Stimulation on the Number of c–Fos Response Cells and c–Fos Expression in the Global Ischemic Rats

INTRODUCTION

The striatum is composed of the caudate and putamen of the basal ganglia. The basal ganglia, an aggregate of nuclei located deep within the cerebral cortex is in charge of habitual learning and is the foundation of clear memory(1).

After brain damage, c–Fos mRNA and protein quickly and temporarily accumulate in the area where the overall lesion of the cerebral cortex occurs(2,3). c–Fos protein has been reported to be related with biological phenomena like cell cycle progression and tumorigenesis(4), and the increase of c–Fos activation has been reported in developing brains, the central nervous system, megakaryocytes, and hematopoietic cells(5,6,7,8).

Global Ischemia attacks cause biochemical changes in brain tissue and increases the expression of neuron genes within minutes of occurring, and the genes displaying such a rapid response are referred to as immediate early genes(9). An immediate early antigen, c–Fos is an important early marker in the process related to stress, learning, and memory, and various studies use it in screening inspections that reflect nerve activity(10).

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Global Ischemia attacks cause biochemical changes in brain tissue and increases the expression of neuron genes within minutes of occurring, and the genes displaying such a rapid response are referred to as immediate early genes(9). An immediate early antigen, c–Fos is an important early marker in the process related to stress, learning, and memory, and various studies use it in screening inspections that reflect nerve activity(10). c–Fos is expressed immediately following ischemic attacks, and after forming activator protein–1(AP–1) in combination with several Jun-type protein, takes effect as a transcription factor within cells that control the transcription of specific genes(11). It is also known to be related to synaptic plasticity and apoptosis in damage from ischemia or external injury(12). In other words, c–Fos quickly induces early genes and their transcription factors specifically in CA1 regions after ischemic injury, and c–Fos induction is observed as functioning as a cause that accelerates neuronal apoptosis after ischemic injury(13).

Therefore, the present study attempts to find a more active approach from an electrotherapeutic perspective toward neuronal apoptosis resulting from early ischemic brain damage, by researching
neuronal c-Fos protein changes in the striatum region after applying needle electrode electrical stimulation with the purpose of finding out how it affects the c-Fos expression of the striatum in cerebral ischemia induced rats.

METHODS

Subjects

The present study used eight week old male SD rats (Orient Bio Inc., Seongnam, Korea) weighing around 300g, with no specific pathogens, which were adapted to the laboratory environment for one week or longer. The temperature and humidity of the laboratory were maintained at 22±1℃ and 45∼55% respectively at all times.

Ischemia induction model and NEES

The experimental animals were under anesthetics with 3% isoflurane (JW Pharmaceutical Corporation, Korea) and after conducting a 2cm median incision to the bilateral carotid artery on the median line of the neck, the vagus nerve was separated to expose the carotid artery and completely ligate the bilateral carotid artery with non-absorbable suture, which was maintained for five minutes, after which the suture was removed so that the blood could be reperfused.

The study was carried out by distinguishing a control group without induced carotid artery occlusion, a global ischemia (GI) group with induced carotid artery occlusion, and an needle electrode electrical stimulation (NEES) group that underwent NEES after induced GI. After 12, 24, and 48 hours of carotid artery occlusion, the GI group recovered from an anesthesia of 3% isoflurane for 30 minutes, while the NEES group underwent NEES using a needle electrode electrical stimulator (PG6, ITO, JAPAN, 9V) on acupuncture point ST36 (Joksamri) on the left and right sides of the rat for 30 minutes, simultaneously with the 3% isoflurane anesthesia. NEES was conducted at an alternating current of 2Hz, and the intensity was adjusted according to the muscle movement around the stimulated area of the rat.

Immunohistochemistry

The study conducted an immunohistochemical test by selecting striatum regions of the model rat which was GI induced by carotid artery occlusion, Frozen in 4% paraformaldehyde, the brain was cryostatically sectioned, using a free-floating method with a Vectastain avidin–biotin complex (ABC) Kit (Vector, USA). The dissected tissue was washed three times for five minutes with 0.1M Phosphate Buffered Saline (PBS), and blocked (peroxidase blocking, 0.1M PBS) with 1% H2O2 after which it was left alone at room temperature for 30 minutes in 2% normal goat serum, Then it was DAB color developed and placed on a tissue slide using a 1% gelatin solution, after which it was dried for about two hours in a dry oven. Next, it underwent a process of dehydration and clearing and then loaded using a permount (Fisher, USA) solution.

Western blotting

The extracted striatum is divided by each region and is crushed homogeneously using a homogenizer. Then, it undergoes centrifugation at 1000 rpm to discard the supernatant, after which it is wash twice using 2mL of cold PBS. The quantified protein is mixed with lysis buffer and sample buffer (60 mM tris; pH 6.8, 10% glycerol, 2% sodium dodecylsulfate (SDS), .01% bromophenol blue) in order to equate the amount of protein and then boiled for five minutes on a 100℃ heat block after which samples were collected by centrifugation. Next, a separating gel (12%~15%) was created by mixing 30% polyacrylamide mix, deionized water, 1.5 M tris (pH 8.8), 10% SDS, 10% ammonium persulfate created that day, and Tetramethylethylenediamine (TEMED). It was then cleaned and poured into an assembled electrophoresis glass plate to solidify into a gel. The stacking gel was mixed with 30% polyacrylamide mix, deionized water, 1 M tris (pH6.8), 10% SDS, 10% ammonium persulfate created that day, and TEMED and poured onto the separating gel to form the completed gel.

The next day, after the blocking solution was removed, the primary antibody identical to the antibody used in immunostaining was diluted a thousand fold in 5% skim milk, the solution upon which, a thin coating was inserted and reacted for an hour. This was then washed three times for ten minutes in Tween 20/Tris–buffered saline (TTBS) solution, and a thin coating was inserted into the solution created by diluting a thousand fold the
secondary antibody in 5% skim milk to induce a reaction for one hour. After the solution was removed, it was washed three times ten minutes each time with TTBS. ECL kit solutions A and B were mixed well at a ratio of 40:1 and soaked with the thin coating and made to react for a minute, after which the thin coating was placed on a cassette and exposed to X-ray film. After it was exposed for a fixed period of time, it was expressed and its band was confirmed and then it was fixed by containing it in a fixator. After the fixing was finished, it was washed in running water and dried to be scanned so that the optical density of each band could be measured with a bio-rad.

Data analysis

All data collected in the present study was analyzed with a statistics processing program (SPSS 12.0K/PC) by using a computer after encoding. In order to find out the effect on the control group, the stroke induced group, and the treatment group after stroke induction, the study analyzed the differences between them by setting the level of significance at \( \alpha = 0.05 \) and by using ANOVA analysis.

RESULTS

The Change in the Number of c-Fos response cells in the Striatum

After ischemia induction, the number of c-Fos response cells in the striatum was revealed to be 115.67 ± 5.03 for the control group, while it was 202.67 ± 12.01 and 178.33 ± 7.37 respectively for the GI group and the NEES group 12 hours after ischemia induction as shown by Fig. 1. This was a significant decrease (\( p < 0.05 \)) for the NEES group (Joksamri) when compared to the GI group.

Change in c-Fos Protein Expression in the Striatum

The c-Fos expression in rat striatum after ischemia induction was 44.21 ± 1.40 for the control group, while it was 76.69 ± 2.81 and 67.56 ± 4.16 respectively for the GI group and the NEES group (Joksamri) 12 hours after ischemia induction, as shown by Fig. 2. This was a decrease for the NEES group in comparison to the GI group, c-Fos expression after 24 hours of ischemia induction was 86.38 ± 5.31 for the GI group (Joksamri) and 58.06 ± 4.00 for the NEES group (Joksamri), which shows an extremely significant decrease (\( p < 0.01 \)) for the NEES group (Joksamri) when compared to the GI group.
The basal ganglia plays an important role in cognitive function by connecting with the prefrontal cortex. Strokes are acute neurological injuries that are caused by blood supply disorder in the brain, and more than 80% of these are caused by ischemia. Global ischemia causes selective neuronal damage and such cell damage features a delayed quality in that it only becomes considerable a few days after the ischemia (14,15). The basal ganglia area, as a result of ischemic attack, displayed increase in protein expression in terms of c-Fos change resulting from GI (16), and the study on the apoptosis of the cerebral ischemia animal model reported a high increase of Immunomodulating activity of the c-Fos in the two hour ischemia group as a result of the immunohistochemical staining of the c–Fos (17). The c–Fos expressed during ischemia is combined with several Jun type proteins and forms (18) the activator protein–1 (AP–1), which is an intracellular transcription, by which it controls major physiological functions like cell proliferation and differentiation, organogenesis, cell death and stress response (19). During the inadequate expression of intracellular material resulting from a harmful environment like cerebral ischemia or injury, control over multiple transcription material and post transcription material may manage the intracellular environment (20,21) and get involved in apoptosis (22). Also, studies have reported that cerebral c–Fos expression has been decreased after conducting NEES on increased c–Fos expression resulting from ischemia (23).

The present study confirms apoptosis during NEES on cell numbers responding to anti-c-Fos and the c-Fos expression in increased hippocampus and striatum resulting from ischemia, which was identical to the expression in the cerebrum of prior studies. The number of striatum c–Fos response cells after GI induction significantly decreased (p<.05) after 12 hours of the induction for the NEES group in comparison to the GI group, and the number of c–Fos response cells 24 hours after ischemia induction significantly decreased (p<.05) for the NEES group when compared to the GI group. The number of c–Fos response cells 48 hours after ischemia induction underwent an extremely significant decrease (p<.01) for the NEES group compared to the GI group. In terms of change in c–Fos protein expression in the striatum, c–Fos expression 12 hours after ischemia induction was decreased more for the NEES group than the GI group. After 24 hours, the NEES group underwent an extremely significant decrease (p<.01) in expression compared to the GI group. After 48 hours, the NEES group significantly decreased (p<.05) in expression when compared to the GI group.

**DISCUSSION**

The basal ganglia plays an important role in cognitive function by connecting with the prefrontal cortex. Strokes are acute neurological injuries that are caused by blood supply disorder in the brain, and more than 80% of these are caused by ischemia. Global ischemia causes selective neuronal damage and such cell damage features a delayed quality in that it only becomes considerable a few days after the ischemia (14,15). The basal ganglia area, as a result of ischemic attack, displayed increase in protein expression in terms of c–Fos change resulting from GI (16), and the study on the apoptosis of the cerebral ischemia animal model reported a high increase of Immunomodulating activity of the c–Fos in the two hour ischemia group as a result of the immunohistochemical staining of the c–Fos (17). The c–Fos expressed during ischemia is combined with several Jun type proteins and forms (18) the activator protein–1 (AP–1), which is an intracellular transcription, by which it controls major physiological functions like cell proliferation and differentiation, organogenesis, cell death and stress response (19). During the inadequate expression of intracellular material resulting from a harmful environment like cerebral ischemia or injury, control over multiple transcription material and post transcription material may manage the intracellular environment (20,21) and get involved in apoptosis (22). Also, studies have reported that cerebral c–Fos expression has been decreased after conducting NEES on increased c–Fos expression resulting from ischemia (23).

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CONCLUSIONS

The number of striatum c-Fos response cells after GI induction significantly decreased after 12, 24, 48 hours of the induction for the NEES group in comparison to the GI group. In terms of change in c-Fos protein expression in the striatum, c-Fos expression 12 hours after ischemia induction was decreased more for the NEES group than the GI group. After 24 hours, the NEES group underwent an extremely significant decrease in expression compared to the GI group. After 48 hours, the NEES group significantly decreased in expression when compared to the GI group.

Considering the research results above, NEES delays the expression period of c-Fos, which accelerates apoptosis during early ischemia, and thus delays and suppresses apoptosis. The present study believes that more caution and in-depth research is required to apply the results obtained in animal experiment to patients suffering from ischemia as a result of cerebrovascular injury.

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REFERENCES