

Effects of NEES on PARP Expression in the Corpus Striatum in Rats Induced with Transient Global Ischemia

Ischemia, the leading cause of strokes, is known to be deeply related to synaptic plasticity and apoptosis in tissue damage due to ischemic conditions or trauma. The purpose of this study was to research the effects of NEES (needle electrode electrical stimulation) in brain cells of ischemia-induced rat, more specifically the effects of Poly[ADP-ribose] polymerase (PARP) on the corpus striatum. Ischemia was induced in SD mice by occluding the common carotid artery for 5 minutes, after which blood was re-perfused. NEES was applied to acupuncture points, at 12, 24, and 48 hours post-ischemia on the joksamri, and at 24 hours post-ischemia on the hapgok. Protein expression was investigated through PARP antibody immunoreactive cells in the cerebral nerve cells and western blotting. The number of PARP reactive cells in the corpus striatum 24 hours post-ischemia was significantly ($p < .05$) smaller in the NEES group compared to the global ischemia (GI) group. PARP expression 24 hours post-ischemia was very significantly smaller in the NEES group compared to the GI group. Results show that ischemia increases PARP expression and stimulates necrosis, making it a leading cause of death of nerve cells. NEES can decrease protein expression related to cell death, protecting neurons and preventing neuronal apoptosis.

Key words: *Ischemia; Necrosis; Needle Electrode Electrical Stimulation; Stroke; Joksamri; Hapgok*

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INTRODUCTION

Ischemia, the leading cause of strokes, is known to be deeply related to synaptic plasticity and apoptosis in tissue damage due to ischemic conditions or trauma(1). There are two kinds of cell death: necrosis and apoptosis. Necrosis is a passive death that occurs in the central region of brain damage following ischemia. Poly(ADP-ribose) polymerase (PARP) is activated when various enzymes within the cell nucleus cause damage to DNA, resulting in necrosis(2, 3). It also plays a role in cell differentiation and genetic expression(4). PARP conserves DNA through a process in which ADP-ribose is passed on to histones and various other nucleonic proteins, including itself(5). PARP uses NAD⁺ as a supplier of ADP-ribose(6), and cell death occurs when PARP is over-activated, and ATP and NAD⁺ are overused, resulting in loss of energy(7, 8, 9, 10, 11, 12).

Continuous ischemic conditions in the brain cause lack of collateral circulation, resulting in the entire brain suffering acute cerebral infarct and a high

mortality rate(13). This kind of permanent damage does not occur if the ischemia is temporary(5–10 minutes), and is followed immediately by reperfusion; however, delayed neuronal death—loss of neurons in specific areas such as the hippocampus or basal ganglia has been observed 3–4 days post ischemia(13, 14).

The positive effects of an oriental medicine approach in the treatment of diseases have already been established. Oriental medicine uses a combination of pharmaceuticals, puncture therapy, and physical methods(15). The treatment effects of electro-puncture vary according to stimuli conditions—the wave pattern and length of stimulation affect activation of serotonin neurons(16). Unlike other puncture methods, electropuncture has the advantage of continuous stimulation as well as allowing for objective adjustment of the amount of stimulation given, making it an even more effective tool in the treatment of debilitating illnesses such as neural pain or paralysis(15).

This study applied NEES to the joksamri and hapgok

areas, acupuncture points that affect brain cell activation, following induction of ischemia in the brain. The corpus striatum is deeply related to human cognitive abilities and motor functions, making it an important part of daily life. The amount of PARP expression in slices of the corpus striatum was investigated in order to research the effectiveness of NEES on acupuncture points in preventing necrosis.

METHODS

Subjects

Subjects were 8 week-old, specific pathogen free, male SD rats which had been allowed to adjust to the laboratory environment for over 1 week and weighed approximately 300g(Orient BIO, Co., Gapyung, South Korea). Appropriate amounts of food and water were given, and the laboratory was kept at $22\pm 1^{\circ}\text{C}$ and 45–55% humidity.

Ischemia Induction Model and NEES

Subjects were anesthetized using 3% isoflurane (Choongwae Pharma Corporation, Korea). Incisions 2cm in length were made in the center of both common carotid arteries, the vagus nerves were separated, and circulation in both arteries was completely cut off using non-absorbent thread. Five minutes later, the thread was removed to allow the reperfusion of blood.

The subjects were divided into the control group(no closing of the common carotid artery), the global ischemia(GI) group(common carotid artery closed), and the NEES group(NEES treatment applied after GI induction). Subjects in the GI group were allowed to recover for 30 minutes at 12, 24, and 48 hours after induction of GI with 3% isoflurane as an anesthetic. Subjects in the NEES group were given NEES treatment by using a needle electrode electrical stimulator(PG6, ITO, Japan, 9V) at the right and left Joksamri(ST36), Hapgok(LI 4) for 30 minutes in concurrence with anesthesia with 3% isoflurane at 12, 24, and 48 hours after induction of GI. NEES was done using a 2Hz current, and strength was adjusted according to muscle movement around the stimulation point.

Immunohistology

Immunohistological studies were performed on striatum tissue samples of GI-induced rats. Fixed

brain tissue was freeze sectioned using 4% paraformaldehyde, and the free-floating method and a Vectastain ACB Kit(Vector, USA) was used. The microtomed tissue was washed 3 times for 5 minutes each with 0.1M PBS, and blocking was performed using 1% H_2O_2 (peroxidaseblocking, 0.1MPBS). The samples were then left in 2% normal goat serum for 30 minutes at room temperature. Anti-PARP was used as the primary anti-body. After leaving the primaryanti-body to react for 3days at 4°C , it was washed 3 times for 5 minutes each with 0.1M PBS, after which it was left to react with the secondary anti-body for 1 day at 4°C . After diaminobenzidine (DAB, Sigma, USA) color development, tissue samples were put onto slides using 1% gelatin, dried in a dry oven for approximately 2 hours, dehydrated and made clear, then mounted using permount solution (Fisher, USA).

Western Blotting

The removed striatum was divided according to area and evenly broken down using a homogenizer. It was centrifuged at 1000rpm and the supernatant was discarded, after which it was washed twice with cold Phosphate Buffered Saline(PBS). After centrifuging, a Protein assay kit(Bio-rad) was used to measure the optical density of the protein at 750nm. The quantified protein was mixed with a lysis buffer and sample buffer(60mM tris; pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue) in order to even the amount of protein, then boiled at 100°C for 5 minutes on a heat block. This was centrifuged and the specimen collected. A separating gel(12–15%) was made by pouring into a clean electrophoresis glass plate a combination of 30% polyacrylamide mix, thrice distilled water, 1.5M tris(pH 8.8), 10% SDS, 10% ammonium persulfate(produced that day), and TEMED. 10x stock of electrophoresis running buffer was made by melting 30.0g tris base, 144g glycine, and 10g SDS into 1L. 10–20 μL of the quantified protein was added, and 100V was run for approximately 1 hour. The transfer buffer was made by adding 3.03g tris-base, 14.63g glycine, and 200mL methanol to 1L. It was stored at 4°C before use. The thin membrane was washed with a Tween/Tris Buffered Saline(TTBS) solution(pH 7.5) composed of 200mM tris-base, 1.54M NaCl, thrice-distilled water, and 20 tween. It was then put into 5% lac defloratum and left for a day at 4°C .

The next day, after removing the blocking solution, a primary anti-body identical to the one used in immune-staining was reacted for 1 hour with the thin

membrane put into a solution made by diluting 5% lac defloratum to 1000 times its original concentration. This was then washed 3 times with TTBS solution for 10 minutes each. A secondary anti-body was reacted for 1 hour with the thin membrane put into a solution made by diluting 5% lac defloratum to 1000 times its original concentration. After removing the solution, this was then washed 3 times with TTBS solution for 10 minutes each. Solutions A and B from the ECL kit were well combined at a ratio of 40:1 and this was used to wet the thin membrane. After 1 hour, the thin membrane was put on a cassette and photo-sensitized using x-ray film. After photo-sensitizing for a period of time, expression bands were checked and a fixator was used for fixing. After fixing, the bands were washed in clean running water, dried, scanned, and a concentration meter(Bio-rad) was used to measure the optical density of each band.

Data Analysis

All data collected in this study were encoded and analyzed using a computerized statistical analysis program(SPSS 12.0K/PC). In order to find out the effects of the control group, global ischemia group, and NEES group, the level of significance was set to $\alpha = .05$. ANOVA was used to analyze the differences between the three groups.

RESULTS

Changes in the Number of PARP Reactive Cells in the Corpus Striatum

The number of PARP reactive cells in the corpus striatum following ischemia can be seen in Figures 1 and 2. PARP reactive cells in the control group was 121.67 ± 7.02 , whereas those in the GI group and NEES(ST 36) group 12 hours post-ischemia were 206.67 ± 19.14 and 184.67 ± 7.02 respectively, showing a significant($p < .05$) decrease in the NEES group compared to the GI group. PARP reactive cells in the NEES(ST 36) group and GI group 24 hours post-ischemia were 226.00 ± 15.52 and 172.00 ± 4.58 respectively, showing a decrease in the NEES(ST 36) group compared to the GI group. PARP reactive cells in the NEES(ST 36) group and GI group 48 hours post-ischemia were 174.00 ± 5.00 and 148.33 ± 7.02 respectively, showing a decrease in the NEES(ST 36) group compared to the GI group.

PARP reactive cells in the NEES(LI 4) group and GI

group 24 hours post-ischemia were 226.00 ± 15.52 and 186.67 ± 12.90 , showing a very significant($p < .01$) decrease in the NEES(LI 4) group compared to the GI group. In a comparison between acupuncture points, the number of PARP reactive cells in the NEES(ST 36) group was 172.00 ± 4.58 and that in the NEES(LI 4) group was 186.67 ± 12.90 , showing no significant difference.

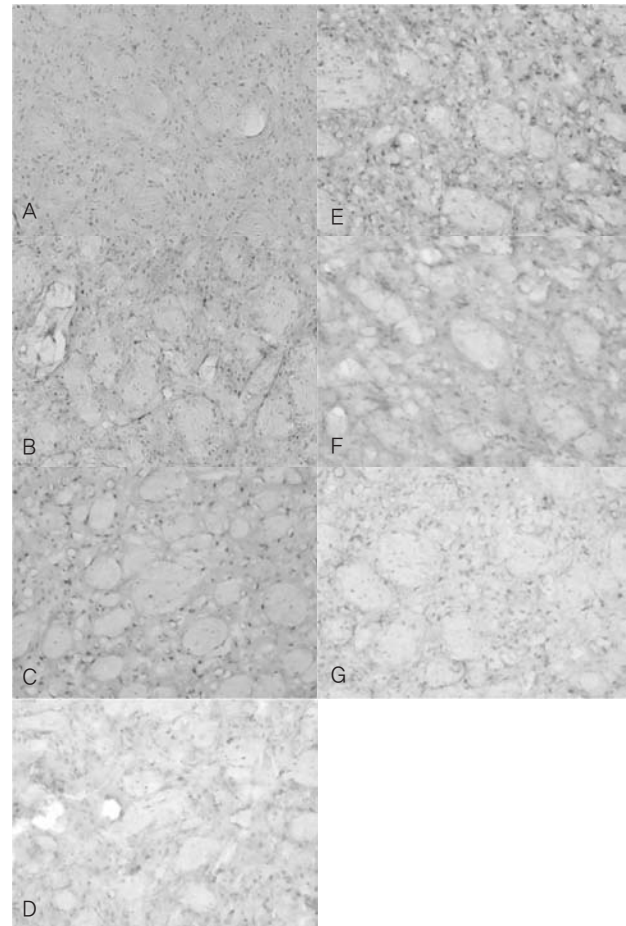


Fig. 1. Effect of needle electrode electrical stimulation on PARP immunoreactive cells in rat striatum after transient global ischemia.

- (a) Control= no surgical procedure(X 200),
- (b) GI= 12h after transient global ischemia(X 200),
- (c) GI= 24h after transient global ischemia(X 200),
- (d) GI= 48h after transient global ischemia(X 200),
- (e) NEES= needle electrode electrical stimulation at 12h after transient global ischemia(X 200),
- (f) NEES= needle electrode electrical stimulation at 24h after transient global ischemia(X 200),
- (g) NEES= needle electrode electrical stimulation at 48h after transient global ischemia(X 200).

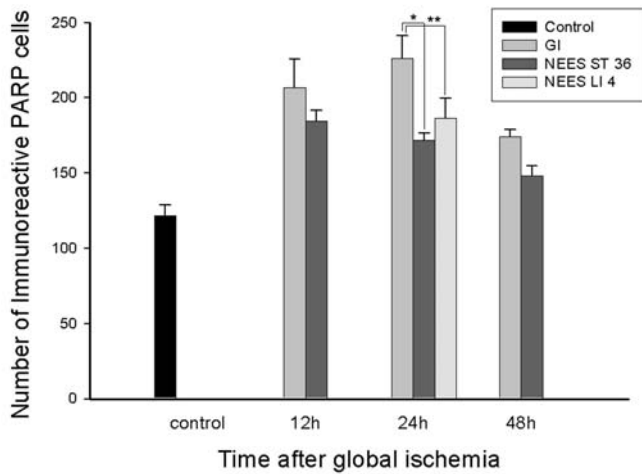


Fig. 2. Effect of needle electrode electrical stimulation on PARP immunoreactive cells in rat striatum after transient global ischemia. Control= no surgical procedure; GI= transient global ischemia; NEES ST 36= needle electrode electrical stimulation at Joksamri, NEES LI 4= needle electrode electrical stimulation at Hapgok. Values are the mean±SD. * $p<0.05$, ** $p<0.01$

Changes in PARP Protein Expression in the Corpus Striatum

PARP expression in the corpus striatum following ischemia can be seen in Figure 3. PARP expression in the control group was 84.42 ± 4.00 , and those in the GI group and NEES(ST 36) group 12 hours post-ischemia were 194.06 ± 3.07 and 190.64 ± 4.82 respectively, showing a decrease in the NEES(ST 36) group compared to the GI group. PARP expression in the GI group and NEES(ST 36) group 24 hours post-ischemia were 190.36 ± 5.16 and 153.55 ± 6.71 respectively, showing a very significant ($p<0.01$) decrease in the NEES(ST 36) group compared to the GI group. PARP expression in the GI group and NEES(ST 36) group 48 hours post-ischemia were 161.62 ± 8.58 and 117.15 ± 12.78 respectively, showing a decrease in the NEES(ST 36) group compared to the GI group. PARP expression in the GI group and NEES(LI 4) group 24 hours post-ischemia were 190.36 ± 5.16 and 165.92 ± 4.88 respectively, showing a very significant ($p<0.01$) decrease in the NEES(LI 4) group compared to the GI group. In a comparison between acupuncture points, PARP expression in the

NEES(ST 36) group was 153.55 ± 6.71 and that in the NEES(LI 4) group was 165.92 ± 4.88 , showing a very significant ($p<0.01$) decrease in the Joksamri compared to the Hapgok area

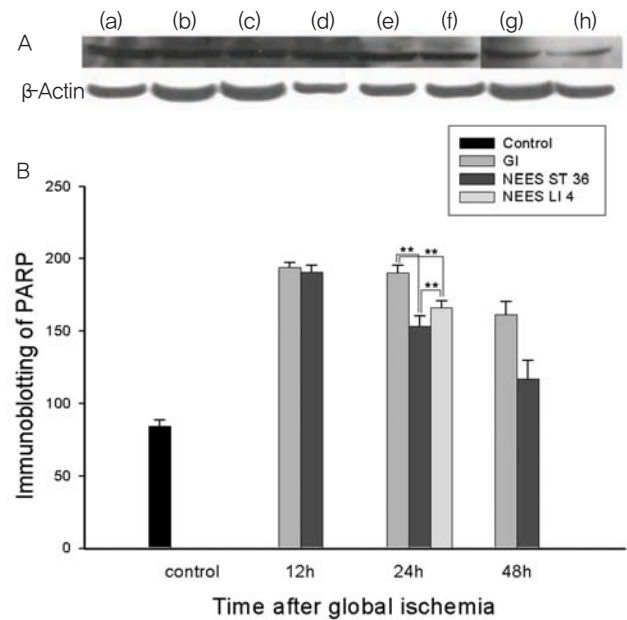


Fig. 3. Changes of PARP expression in rats striatum after transient global ischemia.

- A Total PARP protein was extracted from striatum
 (a) Control group,
 (b) 12h after transient global ischemia,
 (c) needle electrode electrical stimulation(Joksamri) at 12h after transient global ischemia,
 (d) 24h after transient global ischemia,
 (e) Needle electrode electrical stimulation(Joksamri) at 24h after transient global ischemia,
 (f) Needle electrode electrical stimulation(Hapgok) at 24h after transient global ischemia,
 (g) 48h after transient global ischemia,
 (h) needle electrode electrical stimulation(Joksamri) at 48h after transient global ischemia.
- B Results are expressed as density. Control= no surgical procedure; GI=transient global ischemia; NEES ST 36= needle electrode electrical stimulation at joksamri, NEES LI 4= needle electrode electrical stimulation at Hapgok. ** $p<0.01$.

DISCUSSION

It has been reported that occluding the central cerebral artery results in PARP creation and apoptosis inducing factor expression(17). This study investigated the changes in PARP following GI, with results showing the greatest amount observed in the GI group 24 hours post-ischemia in the corpus striatum. These results concur with previous studies that have significantly($p < .05$) decrease the number of PARP reactive cells in both the joksamri and hapgok areas 24 hours post-ischemia, compared to the GI group. Changes in PARP expression was significantly($p < .01$) decreased in the NEES group compared to the GI group 24 hours post-ischemia. These results show that the effects of NEES on PARP immunoreactive cells were greatest 24 hours post-ischemia.

A broad activation of PARP occurs during ischemia, resulting in hyperactivity in necrotic tissue death. This is also the causative factor in the pathological cell death process(18). 3-aminobenzamide is widely used as a PARP inhibitor and decreases the volume of cerebral infarct caused by transient or permanent occlusion of the central cerebral artery(19). 3, 4-dihydro 5-[4±1[1±piperidiny]butoxy]-1 [2H]-isoquinoline is another PARP inhibitor that protects nerves from damage caused by cerebral ischemia(20). PARP inhibitors have been shown to be protected from neurotoxicity, which arises from NO(21), glutamate(22), activated oxygen(23), hydrogen peroxide(24), and peroxyxynitrite(25).

Whether PARP was inhibited by the effects of NEES or the increase of brain-derived neurotrophic factor(BDNF) and BDNF receptor expression is difficult to determine using the results of this study, and further investigation is required.

In conclusion, ischemia has been shown to increase PARP expression and induce necrosis, making it a main causative factor behind neuronal death. NEES can decrease neuronal death by decreasing proteins related to necrosis.

CONCLUSION

The number of PARP reactive cells in the corpus striatum 24 hours post-ischemia was significantly ($p < .05$) smaller in the NEES group compared to the GI group. PARP expression 24 hours post-ischemia was very significantly smaller in the NEES group compared to the GI group. Results show that

ischemia increases PARP expression and stimulates necrosis, making it a leading cause of death of nerve cells. NEES can decrease protein expression related to cell death, protecting neurons and preventing neuronal apoptosis.

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