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The Effects of NEES on PARP Expression and Cell Death in Rat Cerebral Cortex After Ischemic Injury

The majority of strokes are caused by ischemia and result in brain tissue damage, leading to problems of the central nervous system including hemiparesis, dysfunction of language and consciousness, and dysfunction of perception. The purpose of this study was to investigate the effects of Poly(ADP-ribose) polymerase(PARP) on necrosis in neuronal cells that have undergone needle electrode electrical stimulation(NEES) prior to induction of ischemia.

Ischemia was induced in male SD rats(body weight 300g) by occlusion of the common carotid artery for 5 min, after which the blood was reperfused. After induction of brain ischemia, NEES was applied to Zusanli(ST 36), at 12, 24 and 48 hours. Protein expression was investigated using immuno-reactive cells, which react to PARP antibodies in cerebral nerve cells, and Western blotting.

The results were as follows: In the cerebral cortex, the number of PARP reactive cells after 24 hours significantly decreased($p<.05$) in the NEES group compared to the GI group. PARP expression after 24 hours significantly decreased($p<.05$) in the NEES group compared to the GI group. As a result, NEES showed the greatest effect on necrosis-related PARP immuno-reactive cells 24 hours after ischemia, indicating necrosis inhibition, blocking of neural cell death, and protection of neural cells. Based on the results of this study, NEES can be an effective method of treating dysfunction and improving function of neuronal cells in brain damage caused by ischemia.

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

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INTRODUCTION

Strokes are one of the top three leading causes of death in South Korea. Treatment requires a great amount of time, effort and money. Along with a rise in adult diseases due to increased longevity and changes in eating and living habits, the number of stroke incidents that occur each year is also growing(1). The most common cause of stroke is ischemia, caused by ischemic conditions or trauma, and it is known to be related to synaptic plasticity and apoptosis(2).

Neuron apoptosis can occur without severe trauma or direct brain tissue damage. It can occur in long-term and repetitive conditions(3, 4), and necrosis occurs not only in the directly affected area but also in surrounding areas due to secondary effects such as increased intracranial pressure, hypoxia, and ischemia(5). These surrounding areas, which cause cell death, show decreased brain perfusion due to hypoxic and ischemic condition. However, there is a penumbra area in which ATP metabolism and ion homeostasis through cell membrane are maintained(6). Through appropriate treatment after hypoxic and ischemic brain damage, it is possible to recover function in such areas. It is also known that cell death in penumbra areas are mainly caused by cell apoptosis(7). Hypoxic and ischemic brain damage causes external inflammatory reactions, including deposition and accumulation of macrophages, as well as

internal cell damage of the cranial matter. The macrophage deposition in the penumbra areas peaks at 2–3 days after initial damage(8). Apoptosis is found around the center of brain damage and occurs in relatively few areas. It is a programmed cell death that occurs through a set process. Neural apoptosis can occur in long–term and repetitive conditions, without direct brain tissue damage or trauma(3, 4).

Poly(ADP–ribose) polymerase (PARP), which plays a role in necrosis, is an enzyme existing inside the nuclei of various organ cells, including the brain. It is activated when DNA is damaged, acting as a corrector for the damaged DNA(9, 10). In the event of DNA damage, PARP corrects the damage through a process of delivering ADP–ribose to histone and various nucleonic proteins, including itself(11). PARP uses NAD^+ asits source of ADP–ribose(12). If PARP is over–activated, cell necrosis occurs due to energy depletion from over–use of ATP and NAD^+(13, 14, 15, 16, 17, 18).

In this study, NEES was applied to the common carotid artery in ischemia–induced rats to investigate changes in necrosis–related protein PARP in cerebral apoptosis. Effects were observed 12, 24 and 48 hours post–ischemia, in order to find out the efficiency of NEES treatment after stroke.

MATERIALS AND METHODS

Subjects

Subjects were 8 weeks 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±1℃ and 45–55% humidity.

Ischemia Induction Model and NEES

Subjects were anesthetized using 3% isoflurane (Choongwae Pharma Corportation, 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 Zusanli(ST36) 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 frontal cerebral 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, 0.1M PBS). The samples were then leftin 2% normal goat serum for 30 minutes at room temperature. Anti–PARP was used as the primary anti–body. After leaving the primary anti–body to react for 3days at 4℃, 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℃. After DAB color development(Sigma,USA), tissue samples were put onto slides using 1% gelatin, dried in a dry oven for approximately 2 hours, dehydrated and madeclear, thenmounted using permount solution(Fisher, USA).

Western Blotting

The removed cerebrum was divided according to area and evenly broken down using a homogenizer. It was centrifuged at 1000 rpm and the supernatent was discarded, after which it was washed twice with cold 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℃ for 5 minutes on a heat block. This was centrifuged and the specimein collected. A separating gel(12–15%) was made by pouring into a clean elec trophoresis glass plate a combination of 30% poly–acrylamide mix, thrice distilled water, 1.5M tris(pH 8.8), 10% SDS, 10% ammonium persulfate(produced that

day), and TEMED. 10 x stock of electrophoresis running buffer was made by melting 30.0g tris base, 144g glycine, and 10g SDS into 1L. 10-20㎕ 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 methane to 1L. It was stored at 4℃ before use. The thin membrane was washed with a TTBS solution(pH 7.5) composed of 200mM tris-base, 1.54M NaCl, thrice-distilled water, and 20 tween. It was them put into 5% lac defloratum and left for a day at 4℃.

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 min 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 min 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 was 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 α=.05. ANOVA was used to analyze the differences between the three groups.

RESULTS

Changes in Number of PARP Reactive Cells in the Cerebrum

After induction of ischemia, cerebral PARP reactive cells in the mice were as shown in Figures 1 and 2. 12 hours after ischemia, the number of PARP reactive cells in the control group was 76.33±9.61, and those in the GI and NEES groups were 91.67±15.27 and 113.00±4.00 respectively, showing an increase compared to the control group. 24 hours after ischemia, the number of PARP reactive cells in the control group was 77.00±3.61, those in the GI group was 205.67±30.66, and those in the NEES group was 134.67±15.53, showing a significant($p<.05$) decrease compared to the GI group. 48 hours after ischemia, the number of PARP reactive cells in the GI group was 105.67±14.98, showing a great decrease compared to 24 hours, and those in the NEES group were also decreased to 105.67±22.72.

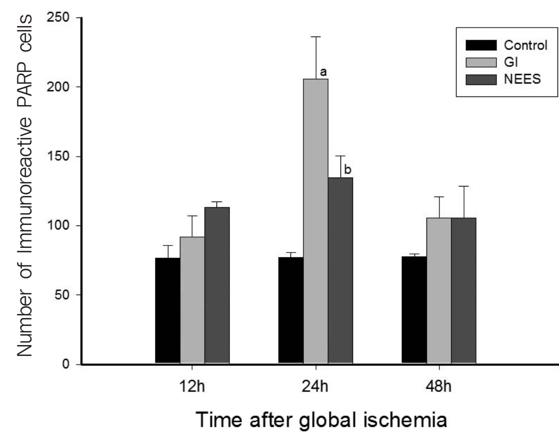
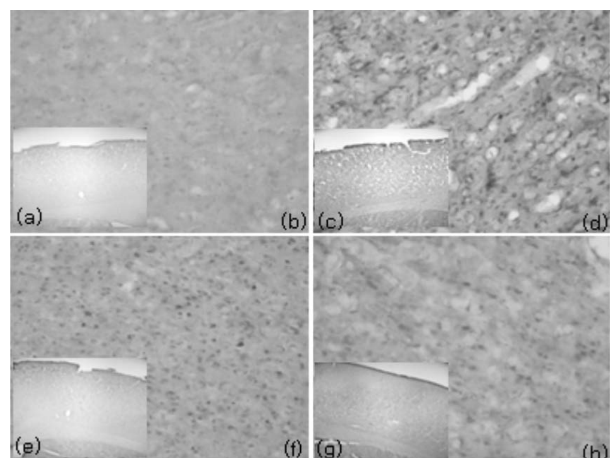


Fig. 1. Effect of needle electrode electrical stimulation on PARP immunoreactive cells in rat cerebrum after transient global ischemia. Control: no surgical procedure, GI: transient global ischemia, NEES: needle electrode electrical stimulation at 12, 24, and 48 hours after transient global ischemia. Values are the mean± SD. Bars with different alphabetic letters in the same times are significantly different(a, b $p<.05$)



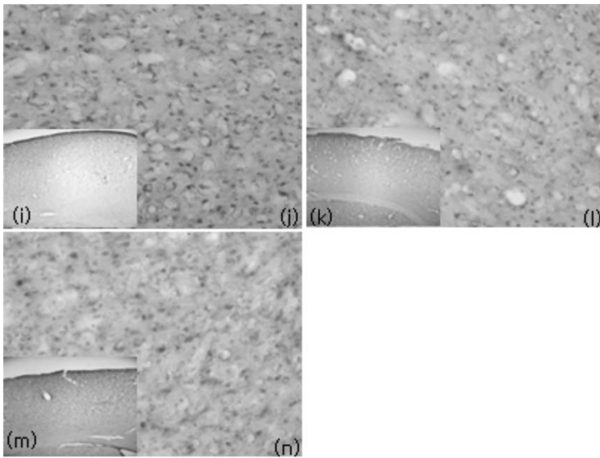


Fig. 2. Effect of needle electrode electrical stimulation on PARP immunoreactive cells in rat cerebrum after transient global ischemia

(a) Control: no surgical procedure(X 40)
(b) Control: no surgical procedure(X 200)
(c) GI: 12h after transient global ischemia(X 40)
(d) GI: 12h after transient global ischemia(X 200)
(e) GI: 24h after transient global ischemia(X 40)
(f) GI: 24h after transient global ischemia(X 200)
(g) GI: 48h after transient global ischemia(X 40)
(h) GI: 48h after transient global ischemia(X 200)
(i) NEES: needle electrode electrical stimulation at 12h after transient global ischemia(X 40)
(j) NEES: needle electrode electrical stimulation at 12h after transient global ischemia (X 200)
(k) NEES: needle electrode electrical stimulation at 24h after transient global ischemia(X 40)
(l) NEES: needle electrode electrical stimulation at 24h after transient global ischemia(X 200)
(m) NEES: needle electrode electrical stimulation at 48h after transient global ischemia(X 40)
(n) NEES: needle electrode electrical stimulation at 48h after transient global ischemia(X 200)

Changes in PARP Protein Expression in the Cerebrum

Changes in PARP protein expression in the cerebrum after induction of ischemia are as shown in Figure 3. 12 hours after ischemia, PARP expression was 116.02±4.00 in the control group, and 152.05±4.60 and 157.67±4.6 in the GI and NEES groups respectively, showing an increase compared to the control group. 24 hours after ischemia, PARP expression was 115.00±3.00 in the control group, 175.07±4.00 in the GI group, and 151.18±4.01 in the NEES group, showing a significant($p<.05$) decrease compared to the GI group. 48 hours after ischemia, PARP expression in the GI group was 193.49±6.50 and 180.00±1.00 in the NEES group, showing a decrease compared to the GI group.

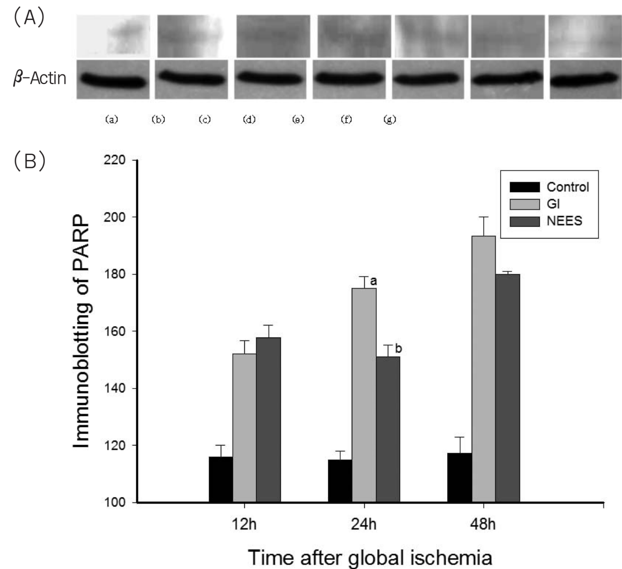


Fig. 3. Effect of needle electrode electrical stimulation on PARP expression in rat cerebrum after transient global ischemia

(A) Total PARP protein was extracted from cerebrum.
(a) Control group
(b) 12h after transient global ischemia
(c) 24h after transient global ischemia
(d) 48h after transient global ischemia
(e) needle electrode electrical stimulation at 12h after transient global ischemia
(f) needle electrode electrical stimulation at 24h after transient global ischemia
(g) needle electrode electrical stimulation at 48h after transient global ischemia

(B) Results are expressed as density.
Control: no surgical procedure, GI: transient global ischemia, NEES: needle electrode electrical stimulation at 12, 24 and 48 hours after transient global ischemia. Values are the mean±SD. Bars with different alphabetic letters in the same times are significantly different(a, b $p<.05$)

DISCUSSION

There are several studies reporting needle treatment to have special effects on the improvement of cerebrovascular accident symptoms, including those caused by ischemia(19, 20, 21), and there are also several studies reporting needle treatment to have special effects on the improvement of cerebrovascular accident symptoms, including those caused by ischemia(22, 23). Such effects of needles are being utilized to promote functional recovery from various impairments, including stroke(10). One of the most powerful effects of needle therapy has been reported to be a speedy recovery from complications related to stroke(24).

PARP is broadly activated during ischemia, and its effects have been clearly shown to hyperactivate necrotic cell death and cause the pathological process of cell death(25). 3-aminobenzamide, commonly used as a PARP antagonist, decreases the volume of cerebral infarction caused by temporary or permanent middle cerebral artery blockage(26). PARP antagonists are also used to protect cells from neuro-toxicity, which contributes to NO(18) and hydrogen peroxide(13).

The results of this study shows that changes in PARP after GI induction are highest in the GI group 24 hours after ischemia is induced in the cerebrum. This concurs with previous study results showing global occurrence during ischemia. The effects of NEES were seen in the number of PARP reactive cells in the cerebrum 24 hours after ischemia, in which the NEES group showed a significant($p<.05$) decrease compared to the GI group. PARP expression significantly($p<.05$) decreased in the NEES group compared to the GI group at 24 hours after ischemia. Based on these results, it can be said that NEES had the greatest effect on necrosis-related PARP immuno-reactive cells at 24 hours after ischemia, indicating necrosis inhibition.

CONCLUSION

Post-ischemic cell death related protein PARP can be decreased using needle electrode electrical stimulation. NEES has been shown to block neural cell death and protects neural cells. The results of this study indicate that NEES can be used to promote function recovery and rehabilitation in ischemia-induced brain damage.

REFERENCES

1. Berger NA. Poly(ADP-ribose) in the cellular response to DNA damage. Radiat Res 1985; 101: 4-15.
2. Cho ZH, Chung SC, Jones JP, Park JB, Park H, Lee HJ, et al. New findings of the correlation between acupoints and corresponding brain cortices using functional MRI. Proc Natl Acad Sci USA 1998; 95: 2670-2673.
3. Endres M, Scott G, Namura S, Salzman AL, Huang PL, Moskowitz MA, et al. Role of peroxynitrite and neuronal nitric oxide synthase in the activation of poly(ADP-ribose) synthetase in a murine model of cerebral ischemia-reperfusion. Neurosci Lett 1998; 248: 41-44.
4. Fisher M. The ischemic penumbra: identification evolution and treatment concepts. Cerebrovasc Dis 2004; 17(suppl 1): 1-6.
5. Gao H, Guo J, Ahao P and Cheng J. The neuroprotective effect of electroacupuncture on focal cerebral ischemia in monkey. Acupunct Electro-Ther Res 2002; 27: 45-57.
6. Ha HC, Snyder SH. Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc Natl Acad Sci USA 1999; 96: 13978-13982.
7. Hausmann R, Biermann T, Wiest I, Tubel J, Betz P. Neuronal apoptosis following human brain injury. Int J Legal Med 2004; 118(1): 32-36.
8. Ikai K, Ueda K, Hayaishi O. Immunohistoche mical demonstration of poly(adenosine diphosphate-ribose) in nuclei of various rat tissues. J Histochem Cytochem 1998; 28: 670-676.
9. Inoue I, Chen L, Zhou L, Zeng X and Wang H. Reoroduction of scalp acupuncture therapy on stroke in the model rats spontaneous hypertensive rat-stroke prone(SHR-SP). Neurosci Lett 2002; 333: 191-194.
10. Jang MH, Shin MC, Koo GS, Lee CY, Kim EH, Kim CJ. Acupuncture decreases nitric oxide synthase expression in periaqueductal gray area of rats with streptozotocin-induced diabetes. Neurosci Lett 2003; 337: 155-158.
11. Karczewski JM, Peters JG, Noordhoek J. Prevention of oxidant-induced cell death in Caco-2 colon carcinoma cells after inhibition of poly(ADP-ribose) polymerase and $Ca2^{+}$ chelation: involvement of a common mechanism. Biochem Pharmacol 1999; 57: 19-26.
12. Kato H, Konure K. Biochemical and molecular characteristics of the brain with developing cerebral infarction. Cellular and Molecular Neurobiol 1999; 19: 93-108.
13. Kim EH, Kim YJ, Lee HJ, Huh Y, Chung JH, Seo JC, et al. Acupuncture increase cell proliferation in dentate gyrus after transient global ischemia in gerbils. Neurosci Lett 2001; 277: 21-24.
14. Kochanek PM, Hallenbeck JM, Polymorphonuc

lear leukocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke. Stroke 1992; 23: 1367-1379.
15. Lee YA. Patients' Lived Experience in Rehabilitating from Stroke. The Kor J Rehabili Nur 2001; 4: 1.
16. Ng I, Yeo TT, Tang WY, Soong R, Ng PY, Smith DR. Apoptosis occurs after cerebral contusions in humans. Neurosurgery 2000; 46: 949-956.
17. Nicoletti VG, Stella AM. Role of PARP under stress conditions; cell death or protection. Neurochem Res 2003; 28: 187-194.
18. Ogura T, Takenouchi N, Yamaguchi M, Matsukage A, Sugimura T, Esumi H. Striking similarity of the distribution patterns of the poly(ADP-ribose) polymerase and DNA polymerase beta among various mouse organs. Biochem Biophys Res Commun 1990; 172: 377-384.
19. Runnerstam M, Bao F, Huang Y, Shi J, Gutierrez E, Hamberger A, Hansson H, Viano D, Haglid K. A model for diffuse brain injury by rotational acceleration, IL Effect on extracellular glutamate Intracranial pressure and neuronal apoptosis. J Neurotrauma 2001; 18(3): 259-273.
20. Schraufstatter IU, Hyslop PA, Hinshaw DB, Spragg RG, Sklar LA, Cochrane CG. Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. Proc Natl Acad Sci USA 1986; 83: 4908-4912.
21. Sharp FR, Lu A, Tang Y, Millhorn DE. Multiple molecular penumbras after cerebral ischemia. J Cereb Blood Flow Metab 2000; 20: 1011-1032.
22. Uchida S, Kagitani F, Suzuki A and Aikawa Y. Effect of acupuncture like stimulation on cortical cerebral blood flow in anesthetized rats. Jpn J Physiol 2000; 50: 495-507.
23. Virag L, Salzman AL, Szabo C. Poly(ADP-ribose) synthetase activation mediates mitochondrial injury during oxidantinduced cell death, J Immunol 1998; 161: 3753-3759.
24. Wallis RA, Panizzon KL, Henry D, Wasterlain CG. Neuroprotection against nitric oxide injury with inhibitors of ADP-ribosylation. Neuroreport 1993; 5: 245-248.
25. Zhang J, Dawson VL, Dawson TM, Snyder SH. Nitric oxide activation of poly (ADP-ribose) synthetase in neurotoxicity. Science 1994; 263: 687-689.
26. Zhang J, Pieper A, Snyder SH. Poly(ADP-ribose) synthetase activation: an early indicator of neurotoxic DNA damage. J Neurochem 1995; 65: 1411-1414.