

The Effect of NEES on the Occurrence of Caspase-3 in the Cerebellum of Rats with Transient Global Ischemia

The cerebellum is known to control balance, equilibrium, and muscle tone. If the cerebellum becomes damaged, the body is unable to retain its balancing functions or involuntary muscle movement. This is why, in stroke patients, there is a high risk of functional disability, as well as a myriad of other disabilities secondary to stroke.

Ischemia was induced in SD mice by occluding the common carotid artery for 5 minutes, after which blood was reperfused. Needle electrode electrical stimulation (NEES) was applied to acupuncture points, at 12, 24, and 48 hours post-ischemia on the joksamri. Protein expression was investigated through caspase-3 antibody immuno-reactive cells in the cerebral nerve cells and Western blotting.

The results were as follows: The number of caspase-3 reactive cells in the corpus cerebellum 12 and 24 hours post-ischemia was significantly ($p < .05$) smaller in the NEES group compared to the GI group. caspase-3 expression 12 and 24 hours post-ischemia was significantly ($p < .05$) smaller in the NEES group compared to the GI group.

Based on these results, NEES seems to have a significant effect on Caspase-3 in the cerebellum in an ischemic state at 12 and 24 hours post ischemia, NEES delays the occurrence of early stage apoptosis-inducing Caspase-3, delaying and inhibiting apoptosis. Further systematic studies will have to be conducted in relation to the application of this study's results on stroke patients.

Key words: Ischemia; Stroke; Apoptosis; Needle Electrode Electrical Stimulation; Joksamri; Caspase-3

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INTRODUCTION

Ischemia, the leading cause of cerebral infarct, is related to synaptic plasticity and apoptosis caused by damage from an ischemic state or trauma(1). The two known mechanisms behind cell death are apoptosis and necrosis.

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Apoptosis is known to occur repeatedly and over long periods of time, even with seemingly mild

degrees of shock and without direct damage to brain tissue(2). Necrosis occurs in areas that have received direct trauma, as well as from secondary factors such as elevated intracranial pressure, hypoxia, and ischemia(3). There is also the penumbra, an area outside the actual place of necrosis or damage, in which hypoxia, ischemia and lack of blood flow cause a decrease in action potential, but ATP metabolism and ionic homeostasis through the cell membrane are maintained(4). Apoptosis is the main mechanism behind cell death in the penumbra, and, with proper treatment following a hypoxic event leading to ischemic brain damage, the penumbra does have potential for rehabilitation(5). Not only does ischemic brain damage cause intrinsic cellular damage to the cerebellar parenchyma, it also causes extrinsic

inflammatory reactions, including accumulation of macrophages, which show a peak in the penumbra at 2–3 days following the initial damage(6).

When ischemia occurs, a protein related to the apoptosis-inducing protein Bax called cytochrome C is isolated from the mitochondria. The isolated cytochrome C activates Caspase-9(7), a central factor in the mechanism behind the cell death process(8), which causes cell death by directly dividing other caspases. Caspase-3 in particular is known to be a main effector caspase which destroys a variety of parenchyma.

This study investigated the changes in Caspase-3 in relation to cell death in the cerebellum in global ischemia-induced rats at 12, 24, and 48 hours post-ischemia, as well as the effects of NEES treatment following ischemic stroke.

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 Needle electrode electrical stimulation(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) 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 cerebellum 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% H₂O₂(peroxidase blocking, 0.1M PBS). 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 primary anti-body to react for 3 days 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 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 made clear, then mounted using permount solution(Fisher, USA).

Western blotting

The removed cerebellum was divided according to area and evenly broken down using a homogenizer. It was centrifuged at 1000 rpm and the supernatant 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°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. 10 x 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

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 antibody 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 antibody 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 Caspase-3 reactive cells in the cerebellum

The number of Caspase-3 reactive cells in the cerebella of ischemia-induced rats at 12 hours post-ischemia was $2.67 \pm .58$ in the control group, 8.00 ± 2.00 in the GI group, and $3.33 \pm .58$ in the NEES group, showing a significant decrease ($p < .05$) in the GI group (Figures 1). The number of Caspase-3 reactive cells at 24 hours post-ischemia was $2.33 \pm .58$ in the control group, 14.00 ± 1.00 in the GI group, and 11.33 ± 1.15 in the NEES group, showing a significant decrease ($p < .05$) in the GI group. The number of

Caspase-3 reactive cells at 48 hours post-ischemia was 9.67 ± 2.52 in the GI group and 8.67 ± 1.15 in the NEES group, showing an insignificant decrease compared to the GI group.

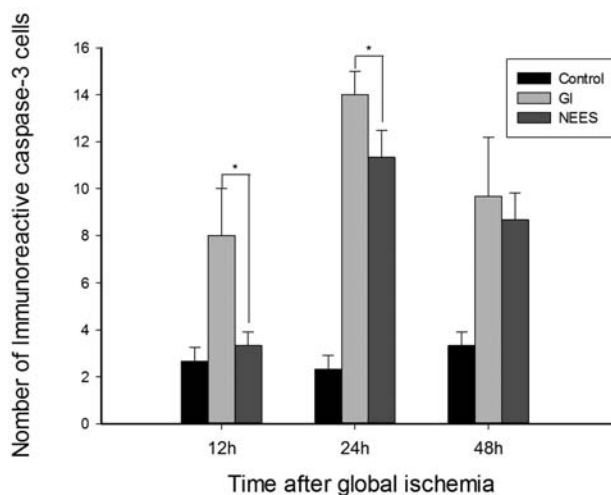


Fig. 1. Effect of needle electrode electrical stimulation on caspase-3 immunoreactive cells in rat cerebellum after transient global ischemia. Control=no surgical procedure; GI=transient global ischemia; NEES=needle electrode electrical stimulation at 12, 24, and 48 hours after transient global ischemia. Values are the mean \pm SD. Bars with different alphabetic letters in the same times are significantly different (* $p < .05$).

Changes in the occurrence of the Caspase-3 protein in the cerebellum

Occurrence of Caspase-3 in the cerebella of ischemia-induced rats at 12 hours post-ischemia was 165.34 ± 3.58 in the control group, 193.76 ± 2.00 in the GI group, and 169.59 ± 4.58 in the NEES group, showing a significant decrease ($p < .05$) compared to the GI group (Figure 2). Occurrence of Caspase-3 at 24 hours post-ischemia was 161.78 ± 5.32 in the control group, 202.63 ± 3.00 in the GI group, and 187.21 ± 3.15 in the NEES group, showing a significant decrease ($p < .05$) compared to the GI group. Occurrence of Caspase-3 at 48 hours post-ischemia in the GI group was 179.99 ± 2.52 , showing a decrease compared to results at 24 hours; however, occurrence in the NEES group was 170.42 ± 3.15 and showed no significant change.

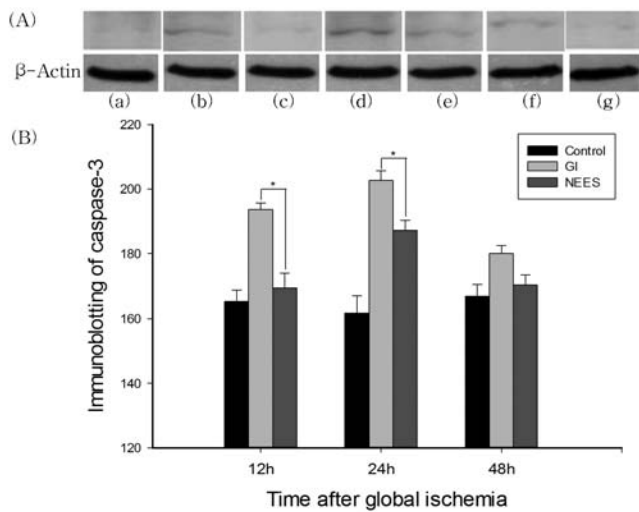


Fig. 2 Effect of needle electrode electrical stimulation on caspase-3 expression in rat cerebellum after transient global ischemia.

- (A) Total caspase-3 protein was extracted from cerebellum.
 (a) Control group,
 (b) 12 h after transient global ischemia,
 (c) 24 h after transient global ischemia,
 (d) 48 h after transient global ischemia,
 (e) needle electrode electrical stimulation at 12 h after transient global ischemia,
 (f) needle electrode electrical stimulation at 24 h after transient global ischemia,
 (g) needle electrode electrical stimulation at 48 h 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 \pm SD. Bars with different alphabetic letters in the same times are significantly different(* $p<.05$).

DISCUSSION

One of the greatest effects of acupuncture therapy is that it can promote speedy recovery from complications related to stroke(9). There are also studies showing that manipulation of certain acupuncture points below the knee may have an effect on blood pressure adjustments(10).

Hyperoccurrence of intercellular Caspase-3 will result in apoptosis(11), and there have been reports that using Caspase-3 activation inhibitors also inhibit apoptosis(12, 13). It has also been reported that the amount of Caspase-9, which becomes ele-

vated in mice with ischemia-induced brain damage, decreases with NEES(14).

An increase in Caspase-3 levels in all areas of the cerebellum following induction of GI was also observed in the present study. Previous studies have reported positive results from NEES therapy at the 12 hour mark post-ischemia, in relation to the effects of NEES on Caspase-3 immunoreactive cells in the ischemic state(15). These results concur with other studies showing that excessive occurrence of Caspase-3 increases cell death, and that ischemia induces an increase in Caspase-9, which, in turn, activates Caspase-3 and results in apoptosis. In regards to the effects of NEES, the number of Caspase-3 reactive cells in the cerebellum 12 hours post-ischemia was 8.00 ± 2.00 in the GI group and 3.33 ± 0.58 in the NEES group, showing a significant decrease compared to the GI group($p<.05$). At 24 hours post-ischemia, the number was 14.00 ± 1.00 in the GI group and 11.22 ± 1.15 in the NEES group, showing a significant decrease as well($p<.05$). Occurrence of Caspase-3 at the 12 hour mark was 193.76 ± 2.00 in the GI group and 169.59 ± 4.58 in the NEES group, showing a significant decrease compared to the GI group($p<.05$). Occurrence of Caspase-3 at the 24 hour mark was 202.63 ± 3.00 in the GI group and 187.21 ± 3.15 in the NEES group, showing a significant decrease compared to the GI group($p<.05$). Based on these results, NEES seems to have a significant effect on Caspase-3 in the cerebellum in an ischemic state at 12 and 24 hours post ischemia. It was concluded that NEES delays the initial occurrence of Caspase-3, resulting in a delay and inhibition of cell death, as well as promotes a decrease in Caspase-3 in early treatment.

In conclusion, NEES delays the occurrence of early stage apoptosis-inducing Caspase-3, delaying and inhibiting apoptosis. Further systematic studies will have to be conducted in relation to the application of this study's results on stroke patients.

CONCLUSION

The number of caspase-3 reactive cells in the corpus cerebellum 12 and 24 hours post-ischemia was significantly($p<.05$) smaller in the NEES group compared to the GI group. caspase-3 expression 12 and 24 hours post-ischemia was significantly($p<.05$) smaller in the NEES group compared to the GI group.

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