The Effect that the Application of Time-Based Electrolysis Has on Acute Ischemia

This neurological damage accelerates the infection reaction of cells and apoptosis at the time of reperfusion after ischemia occurs. BCL-2/BCL-2 allogeneic begeminum has a function of suppressing the apoptosis of cells, and thus it is inferred that the susceptibility of cells to apoptosis is determined by the amount of allogeneic begeminum present which is determined based on the amount of BAX. 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, Hapgok. Protein expression was investigated through BAX antibody immuno-reactive cells in the cerebral nerve cells and Western blotting.

The results were as follows:

In the present study as well, as a result of observation of the change in the number of the BAX reaction cells after the inducement of GI, there was the aspect of most of the BAX reaction cells being observed in the corpus striatum area of the GI group 24 hours after the inducement of ischemia. This revealed the same results as those of previous studies in which the change in the number of BAX reaction cells occurred in all areas while ischemia was in progress. The change in the expression of BAX protein after 24 hours showed that there was a very significant reduction in the NEES group compared to the GI group ($p<.01$). As a result, a greatest amount of change in the number of BAX immunoreactive cells related to apoptosis 24 hours after ischemia appeared in the NEES group. This study that ischemia increases the expression of BAX that induces apoptosis. Thus, it is determined that ischemia is the main cause of the apoptosis of neurons, and this study reveals that low frequency needle electrode electrical stimulation has the effect of blocking the apoptosis of neurons by reducing protein related to the apoptosis of cells that has increased after ischemia has occurred.

Key words: *Ischemia; Apoptosis; Needle Electrode Electrical Stimulation; Joksamri; Hapgok; BAX*

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INTRODUCTION

Ischemic Strokes are categorized as cerebral infarctions and temporary ischemic seizures. In the case that an ischemia of neurons occurs, the supply of oxygen to cells is blocked so that the ATP of microcondria becomes depleted, and acidosis occurs within cells caused by the increase in lactic acid due to anaerobic metabolism. When this condition is maintained for a long period, apopto-

sis of cells occurs. In the case that ischemia and reperfusion occurs within a short period of time, the number of excitatory neurotransmitters of neurons increases. In addition, calcium flows into cells, which causes secondary neurological damage. It is believed that this neurological damage accelerates the infection reaction of cells and apoptosis at the time of reperfusion after ischemia occurs(1). BCL-2/BCL-2 allogeneic begeminum has a function of suppressing the apoptosis of cells,

and thus it is inferred that the susceptibility of cells to apoptosis is determined by the amount of allogeneic begeminum present which is determined based on the amount of BAX(2). Thus, it follows that cells tend to experience apoptosis easily in the case that the amount of BAX relatively increases. And, cells tend to not experience apoptosis easily in the case that the amount of BCL-2 relatively increases. Apoptosis of cells is regulated by the protein BAX that boosts the self-destruction of cells and the protein Bcl-2 that restrains the self destruction of cells. In addition, cytochrome C that has been removed activates caspase 9, which then activates caspase 3 and results in apoptosis of cells(3). BAX is an indicator that determines whether the neurons have been damaged (4). A recent report that states that the apoptosis of neurons due to natural causes was noticeably suppressed in test animals which had BAX genes removed clearly explains the effects of BAX (5).

BAX is one of the gene clusters of bcl-2 which synthesizes pro-apoptotic proteins, and the activity of the transcription of BAX as a transcriptional target is suppressed due to mutant p53. BAX maintains a parallel state by being combined with Bcl-XL, and when a large amount of BAX molecules are produced, the BAX molecules which have been removed become activated and activate a P number ICE/caspases which accelerates apoptosis of cells. Thus, this study researched the change of BAX that is related to the apoptosis of cells within the corpus striatum of the the common carotid artery of mice induced with ischemia of the low frequency needle electrode electrical stimulation (NEES) group and the ischemia inducement(GI) group. In addition, the differences at 12 hours, 24 hours and 48 hours after the inducement of ischemia and the effects of electrolysis after strokes were studied.

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). 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, Hapmok 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 ABC Kit (Vector, USA) was used. 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 striatum 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 μ L 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% lactoflorescence 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% lactoflorescence 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% lactoflorescence 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 photosensitizing 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

The Change in the Number of the BAX Reaction Cells of Corpus Striatum

After the inducement of ischemia, the number of the BAX reaction cells of the corpus striatum of mice as can be seen in Fig. 1 was 67.67 ± 3.05 in the control group. 12 hours after the inducement of ischemia, the number of the BAX reaction cells in the GI group and the NEES group was 109.67 ± 7.57 and 94.67 ± 3.51 respectively, which showed a reduction in the NEES group compared to the GI group. The number of the BAX reaction cells 24 hours after the inducement of ischemia in the GI group and NEES group was 136.67 ± 10.60 and 106.00 ± 3.60 respectively, which showed a significant reduction ($p < .05$) in the NEES group compared to the GI group. 48 hours after the inducement of ischemia, the number of the BAX reaction cells was 97.67 ± 6.11 and 79.67 ± 7.50 in the GI group and NEES group respectively, which showed a significant reduction ($p < .05$) in the NEES group compared to the GI group. In addition, the number of BAX reaction cells 24 hours after the inducement of ischemia was 136.67 ± 10.60 and 104.67 ± 5.50 in the GI group and NEES group respectively, which showed a significant reduction ($p < .05$) in the NEES group compared to the GI group. However, in comparison of spots on the body related to acupuncture, the number of BAX reaction cells 24 hours after the inducement of ischemia was 106.00 ± 3.60 in the NEES group and 104.67 ± 5.50 in the NEES group, in which there was not a significant difference between Joksamri and Hapgok.

Fig. 1. Effect of needle electrode electrical stimulation on BAX 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 $\pm$ SE. * $p<0.05$.

Change in the Expression of BAX Protein of the Corpus Striatum

After the inducement of ischemia, the expression of the BAX reaction cells of the corpus striatum of mice as can be seen in Fig. 2 was 44.35 ± 3.11 in the control group. 12 hours after the inducement of ischemia, the expression of the BAX reaction cells in the GI group and the NEES group was 72.06 ± 3.45 and 67.99 ± 4.32 respectively, which showed a reduction in the NEES group compared to the GI group. The expression of the BAX reaction cells 24 hours after the inducement of ischemia in the GI group and NEES group was 84.34 ± 3.65 and 56.21 ± 5.65 respectively, which showed a very significant reduction ($p<0.01$) in the NEES group compared to the GI group. 48 hours after the inducement of ischemia, the expression of the BAX reaction cells was 71.87 ± 3.79 and 61.32 ± 2.49 in the GI group and NEES group respectively, which showed a very significant reduction ($p<0.01$) in the NEES group compared to the GI group.

In addition, the expression of BAX reaction cells 24 hours after the inducement of ischemia was 84.34 ± 3.65 and 76.24 ± 3.88 in the GI group and the NEES group respectively, in which there was not a significant difference between the two groups. In comparison of spots on the body relat-

ed to acupuncture, the expression of BAX reaction cells 24 hours after the inducement of ischemia was 56.21 ± 5.65 in the NEES group and 76.24 ± 3.88 in the NEES group, in which there was not a significant difference between Joksamri and Hapgok.

Fig. 2 Changes of BAX expression in rats striatum after transient global ischemia.

(A) Total BAX protein was extracted from striatum.

- (a) Control group,
- (b) 12 h after transient global ischemia,
- (c) needle electrode electrical stimulation (Joksamri) at 12 h after transient global ischemia,
- (d) 24 h after transient global ischemia,
- (e) needle electrode electrical stimulation (Joksamri) at 24 h after transient global ischemia,
- (f) needle electrode electrical stimulation (Hapgok) at 24 h after transient global ischemia,
- (g) 48 h after transient global ischemia,
- (h) needle electrode electrical stimulation (Joksamri) at 48 h 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. ** $p<0.01$, * $p<0.05$.

DISCUSSION

Stress in the form of neurotransmitters of brain cells, depolarization, the inflow of calcium and the like cause a biochemical change within cells, and genes which quickly respond to biochemical change are called immediate early genes(IEGs)(6).

BAX is a protein that has similarities with Bcl-2 and forms a heterodimer together with a homodimer or Bcl-2. The expression of BAX increased in the cerebral infarction area at the time of cerebral ischemia in white mice(7), and the expression of the BAX protein of corpus striatum and the number of cells that reacted to Anti-BAX after the inducement of forebrain ischemia increased (Fig. 1, 2).

IEG p53 regulates the ratio of Bcl-2/BAX, and the increase in the proportion of BAX induces apoptosis of cells(8). BAX that is excessively expressed in a diseased state suppresses the cell protection activity of Bcl-2 and thus induces apoptosis of cells(9). Proteins in the Bcl-2 family release cytochrome c from the mitochondria into the cytoplasm, and they regulate apoptosis through the promotion of BAX and the suppression of Bcl-xl(10, 11). Upon the release of cytochrome c into the cytoplasm, caspase that induces apoptosis is activated and apoptosis occurs.

It has been reported that in test animals in which BAX genes were removed, the apoptosis of neurons was noticeably suppressed(12). In addition, upon blocking the middle cerebral artery of white mice, low frequency needle electrode electrical stimulation delayed the expression of BAX (13). In terms of forebrain ischemia, it was shown that there was a neuroprotective effect when low temperature reduced the expression of the BAX protein(14). In the present study as well, as a result of observation of the change in the number of the BAX reaction cells after the inducement of GI, there was the aspect of most of the BAX reaction cells being observed in the corpus striatum area of the GI group 24 hours after the inducement of ischemia. This revealed the same results as those of previous studies in which the change in the number of BAX reaction cells occurred in all areas while ischemia was in progress. In addition, in regards to the effectiveness of NEES, it was shown that the number of BAX reaction cells in the corpus striatum area 24 hours after the inducement of ischemia was significantly reduced in both Joksamri and Hapgok of the NEES group

in comparison to the GI group. The change in the expression of BAX protein after 24 hours showed that there was a very significant reduction in the NEES group compared to the GI group($p<.01$). As a result, a greatest amount of change in the number of BAX immunoreactive cells related to apoptosis 24 hours after ischemia appeared in the NEES group.

As mentioned above, it has been shown in this study that ischemia increases the expression of BAX that induces apoptosis. Thus, it is determined that ischemia is the main cause of the apoptosis of neurons, and this study reveals that low frequency needle electrode electrical stimulation has the effect of blocking the apoptosis of neurons by reducing protein related to the apoptosis of cells that has increased after ischemia has occurred.

CONCLUSION

In the present study as well, as a result of observation of the change in the number of the BAX reaction cells after the inducement of GI, there was the aspect of most of the BAX reaction cells being observed in the corpus striatum area of the GI group 24 hours after the inducement of ischemia. This revealed the same results as those of previous studies in which the change in the number of BAX reaction cells occurred in all areas while ischemia was in progress. The change in the expression of BAX protein after 24 hours showed that there was a very significant reduction in the NEES group compared to the GI group($p<.01$). As a result, a greatest amount of change in the number of BAX immunoreactive cells related to apoptosis 24 hours after ischemia appeared in the NEES group. This study that ischemia increases the expression of BAX that induces apoptosis. Thus, it is determined that ischemia is the main cause of the apoptosis of neurons, and this study reveals that low frequency needle electrode electrical stimulation has the effect of blocking the apoptosis of neurons by reducing protein related to the apoptosis of cells that has increased after ischemia has occurred.

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