Effects of EA Application to GV20 and LI4 on BAX and NF-κB Expression of the SD-Rat’s Hippocampus with Global Ischemia

This study aims to reveal how EA affects BAX and NF-κB involved in cell deaths from global ischemia, and to do this, observes the changes of BAX and NF-κB caused by EA application after transient global ischemia. The experimental method is to give rise to global ischemia and apply EA to 27 SD rats with the particulars of being six–week–old, male, around 300 gram–weighing, and adapted to laboratory environment for more than a week, and divide them into three groups, that is, GV20 EA group(n=9), L14 EA group(n=9), no–treatment Gl group(n=9), and then observe their changes of BAX and NF–κB at the time lapse of 6 hours, 9 hours and 12 hours after ischemia, using western blotting. The numerical decrease of BAX expression at the time lapse of 9 hours after EA application, though not statistically significant, was observed in GV20 EA group and L14 EA group, and the NF–κB expression appeared statistically significant decrease in GV20 EA group and L14 EA group, but the expression was higher in the group with EA application. Therefore, EA application at the early phase of global ischemia is considered to affect BAX and NF–κB and play a positive role in decreasing apoptosis and cell deaths by inflammation.

Key words: Electro–Acupuncture; GV20; LI4; BAX; NF–κB

INTRODUCTION

When cerebrovascular disease takes place in cerebral tissue, blood and oxygen supply will be stopped and it will ischemic necrosis and tissue destruction, resulting in hemiplegia, speech and language disorder, and consciousness disorder and so forth(1). Transient global cerebral ischemia, which occurs more frequently than permanent ischemia, bring forth transient blood circulation disorder in the whole brain and nerve cell damage in the areas sensitive to hypoxia(2). In oriental medicine, researches focus on cerebral blood circulation improvement, betterment of neuro–modulatory function in central nervous system, treatment for central nervous system damage such as cerebral infarction(3–5).

Electro–acupuncture(EA) combining electrode stimulation effect and the merit of acupuncture is used in many countries, When cerebral blood flow increases due to EA application, brain plasticity is activated and surviving cerebral cells around the central area of cerebral infarction rapidly build new neuro–network and replaces the dead cerebral cells function and this is known to help recovering the motor skill of upper and lower limb or language skills(6). Generally, in the treatment of stroke, GV20, GV26, L14, SP6, ST36 are the mainly applied acupuncture points, which are known to have the nerve protection effect(7). When cerebral nerve cells get ischemic injury due to ischemic stroke, there will characteristically arise delayed cell death in a few hours at the shortest or in a few weeks at the latest, This delayed cell death is explained by apoptosis and inflammation.

Rordorf et al, reported that ischemic injury could be divided into reversible injury and irreversible injury, and in nerve cells with reversible injury, expression
of the early genes such as c–eos, c–jun, BCL–2 family would appear(8). BAX is a protein in mitochondria membrane or nuclear membrane and forms dimer within a cell. Furthermore, it is a member of the BCL–2 family and acts by forming a heterodimer in the form of BCL–2 and BCL 2/BAX(9). BCL–2 family transcribes the specific proteins regulating the prescribed cell deaths in various physiological and pathological conditions and though their exact roles are still controversial, BAX, among these, is known to activate and accelerate apoptosis, while BCL–2 is known to inhibit apoptosis(10).

NF–kB is known to be activated in the form of heterodimer of p60 and p65 when in the cytoplasm, heterodimer of p50 and p65 and inhibiting protein IkB kinase is decomposed, and move toward the cell nucleus to accelerate the gene expression of cytokine which induces inflammatory response, INOS · COX–2 adhesion molecule, namely, intracellular adhesion molecule(ICAM) and vascular cell adhesion molecule(VCAM)(11, 12). This NF–kB combines with the promotor area controlling the expression of interleukin(IL)–1β which is a proinflammatory cytokine, IL–6, TNF–α and plays the key role in regulating their transcriptions(13).

There is a consensus among many researchers that in experimental models of various forms of cerebral ischemia, strong expressions of some IEGs(intermediate early genes) including c–fos, c–jun, BAX, NF–kB both within 24 hours and after 24–48 hours are observed(13, 14). However, as there are few studies observing the changes of IEG in hippocampal tissue within 12 hours after EA application, this study observes the early–stage changes of hippocampal tissue due to EA application after transient global cerebral ischemia and examines how EA affects the early IEG.

MATERIALS AND METHODS

Experimental Animals

27 SD(Sprague Dawley) rats were used, after adapted to laboratory environment for more than a week, and their features were around~300g–weighing, 8–week–old, specific pathogen free(SPF), and male (Orient Bio Inc., Located in Gapyeong, Geonggi–do).

Common Carotid Artery Occlusion Model

Common carotid artery occlusion model was used, with global cerebral ischemia induced by common carotid artery ligation as previously reported(15). A rat was anesthetized with 3% isoflurane(Choongwa Pharma Corporation, Korea), the area 2 cm left apart from the very middle line of the neck was incised, the vagus nerve was separated, and common carotid artery was exposed. Then, common carotid artery was completely ligated with the nonresorbable suture and after 5 minutes elapsed, the suture was removed for reperfusion of blood flow. The experiment was conducted with occlusion period set as 6, 9, and 12 hours.

Division of Experimental Groups

The experiment was conducted with the subjects divided as non–EA group which got global ischemia by common carotid artery occlusion but to whom EA stimulation was not applied, L14 EA group and GV20 EA group, which got global ischemia and after 6 hours EA stimulation was applied at L14 and GV20, respectively. Non–EA group and EA groups consisted of 3 rats respectively, and were observed for 6 hours, 9 hours and 12 hours after blood flow reperfusion. Non–EA group was recovered after 6 hours with 30 minutes, 3% isoflurane anesthesia, while EA groups received EA treatment for 30 minutes with anesthesia induction.

1) Non–EA group(Non EA, n=9): When 5 hours and 30 minutes elapsed after induction of global ischemia, only anesthetization was applied, and each brain was extracted at the time lapse of 6 hours, 9 hours and 12 hours, respectively.

2) GV20 EA group(GV 20 EA, n=9): When 5 hours and 30 minutes elapsed after induction of global ischemia, EA at GV20 was applied for 30 minutes under anesthetized condition, and each brain was extracted at the time lapse of 6 hours, 9 hours and 12 hours, respectively.

3) L14 EA group(L14 EA, n=9): When 5 hours and 30 minutes elapsed after induction of global ischemia, EA at L14 was applied for 30 minutes under anesthetized condition, and each brain was extracted at the time lapse of 6 hours, 9 hours and 12 hours, respectively.

Electro–Acupuncture(EA)

With the rats anesthetized with 3% isoflurane, the L14–equivalent area between the first and second interphalangeal joints of the SD rats’ left and right feet and GV20–equivalent area at the center of the parietal bone were located(16). EA stimulation was continuously applied for 30 minutes with the EA
stimulating instruments (PG6, ITO, JAPAN, 5/20 Hz, 1–3 mA) (17) (Fig. 1).

Fig. 1. The facts that are applied at EA in the GV20 and LI4

**Western Blotting**

The extracted brains were divided into parts, and the hippocampus part was crushed with a homogenizer and put into centrifugation at 1000rpm, and after the supernatant being removed, was washed twice in 2ml cold PBS. Here, 200μl of lysis buffer made from 1% Triton X-100, 50mM Tris–HCl (pH 8,0) 150mM NaCl, 1mM EDTA, 1mM EGTA, protease inhibitor cocktail was added, vortexing was done for 10 minutes conducted, and after lysis for some 10 minutes, centrifugation at 4°C, 15000rpm was conducted for 20 minutes. The supernatant being taken after centrifugation, the amount of protein was measured by way of absorption measurement at 70nm with a protein assay kit (Bio-rad).

Samples were collected by mixing lysis buffer and sample buffer (60mM tris: pH6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue) to the obtained protein assays to make equal the contents of protein and then boiling at 100°C heat block for 5 minutes and spinning–down, Separating gels (12%, 15%) were made by mixing 30% polyacrylamide mix and three-times–distilled water, 1.5M tris (pH8.8), 10% SDS, daily–made 10% ammonium persulfate, and TEMED, and then pouring and the solution on a electrophoresis glass plate cleanly washed and assembled and coagulating it into gel. Stacking gel was formed by mixing 30% polyacrylamide mix, three–times–distilled water, 1M tris (pH6.8), 10% SDS, daily made 10% ammonium persulfate, and TEMED, 10 x stock of electrophoresis running buffer was made by dissolving tris base 30.0g, glycine 144g, SDS 10g in 1L, With 10, 20μl of the measured protein loaded, electrophoresis running lasted for 1 hour.

Gel after electrophoresis was transferred to nitrocellulose membrane at 100V for 1 hour. Transfer buffer was made as the one composed of tris–base 3.03g glycine 14.63g, methanol 200ml in 1L, and stored at 4°C. Transferred membrane was washed with TTBS (pH7.5) solution made of 200mM tris–base, 1.54M NaCl, three–times–distilled water and tween 20, and stored overnight at 4°C in 5% skim milk.

After removing the blocking solution, the membrane with the primary antibody (Anti–Bax: Sant acruz, SC–493), Anti–NF–kB (Santacruz, SC–7178) was put, for 1 hour, into the solution diluted 1,000 times with 5% skim milk to induce reactions, and after washing three times with TTBS solution for 10 minutes, the membrane with the second antibody put, for 1 hour, into the solution diluted 1000 times with 5% skim milk to induce reactions. After the removal of the solution, 10 minutes washing were conducted three times, ECL kit (Amersham, USA) solution A and B were mixed by 4:1 and applied to the membrane to induce reaction for 1 minute. After that, the membrane was put on the cassette to be sensitized on the X–ray film. Sensitized for the required time, the film was developed and, after the band in it being identified, fixed in the fixer, After fixing, washing with flowing water and drying were completed, the optical density of the band was scanned and measured with a densitometer (Bio–red).

**Statistical Analysis**

All the collected data, after coding, was analyzed in 2 phases by SPSS 17.0/PC.

In the first phase, descriptive statistics such as frequency, average and standard error were measured to find out the characteristic distributions of the research subjects.

In the second phase, Kruskal Wallis test was conducted at the significance level of α=0.05 to analyze non–EA group, LI4–EA group and GV20–EA group at the time lapse of 6 hours, 9 hours and 12 hours after global ischemia generation.

**RESULTS**

Observing the changes of BAX and NF–kB by western blotting at the time lapse of 6 hours, 9 hours and 12 hours after global ischemia to find out how EA application after transient global ischemia affects the BAX and NF–kB of hippocampus, the following results are obtained.
BAX Western Blot

Table 1. Analysis results of BAX western blotting for each group (hippocampus area/ at the same time), Non EA; no applied at EA; GV20 EA; EA applied at acupoint GV20; L14 EA; EA applied at acupoint L14

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>N</th>
<th>Scores (Rank Sums)</th>
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*$p<.05$

As shown in Figure 3, the expression of BAX at the time lapse of 6 hours after transient global ischemia appeared 26.63±1.36 in non-EA group, 29.10±1.97 in GV20–EA group and 30.87±2.35 in L14–EA group. The expression of BAX at the time lapse of 9 hours was 27.98±2.44, 17.13±1.89 and 24.43±2.12 respectively, and was the lowest in GV20–EA group.

NF–kB Western Blot

As shown in Figure 5, the expression of NF–kB in hippocampus at the time lapse of 6 hours appeared 17.77±1.78 in non–EA group, 29.30±1.11 in GV20–EA group and 28.50±2.35 in L14–EA group respectively. The expression at the time lapse of 9 hours was 9.77±1.36, 7.53±.86 and 14.90±1.85 respectively.

Table 2. Analysis results of NF–kB western blotting for each group (hippocampus area/ at the same time), Non EA; no applied at EA; GV20 EA; EA applied at acupoint GV20; L14 EA; EA applied at acupoint L14

<table>
<thead>
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<th>Group</th>
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*$p<.05$
The expression at the time lapse of 12 hours was 6.70±.82, 4.73±.76, 5.47±1.30 respectively (Fig. 5). The expression of NF-kB showed statistically significant decrease (p<.05) in GV20–EA group and LI4–EA group, while non–EA group didn’t (Table 2).

![Image of western blot](image)

**Fig. 4.** Temporal changes in expression of NF-kB protein in the hippocampus after transient global ischemia. Upper photographs, results of western blot for NF-kB (6, 9, 12 hours after global ischemia) protein. Upper bar graphs show relative levels of β-actin protein expression.

![Graph of related net intensity](image)

**Fig. 5.** Comparison of NF-kB lane by western blotting in each group in the hippocampus (6, 9, 12 hours after global ischemia). Non EA: no applied at EA; GV20 EA: EA applied at acupoint GV20; LI4 EA: EA applied at acupoint LI4.

**DISCUSSION**

It is reported that apoptosis occurs even in the state of long-term and repetitive brain shock which is not so serious as to cause the direct injury of cerebral tissue (18, 19), while necrosis occurs not only in the directly shocked area but also form the secondary factors such as increased intracranial pressure (ICP), hypoxia and ischemia (20). Besides this necrosis area, the apoptosis–generating periphery of brain injury has no action potential owing to blow flow decrease in hypoxia–ischemia (21). It is known that this periphery is a restorable area by appropriate treatment after hypoxia–ischemia brain injury, and the main type of cell death in this area is apoptosis (22). Hypoxia–ischemia brain injury generates not only the inner nerve cell damage but also the external inflammation including the trapping and accumulation of macrophage, and this macrophage is trapped intensively around the periphery in two or three days (23).

It is known that Bcl–2, which is a typical protein expressed by Bcl–2 gene family regulating the programmed cell death in the physiological or pathological state, facilitates cell survival, while BAX facilitates cell death (24). Chen et al. reported in the model of white rat’s local global ischemia that in case of ischemic damage being sub–lethal, say, not so serious as to generate cerebral infarction, the expression of Bcl–2 increases in the area generating ischemia, while in case of ischemic damage being lethal enough to cause cerebral infarction, the expression of Bcl–2 occurs in the vascular endothelial cells and smooth muscles around infarction area (25). Jo et al. observed the expression of several genes involved in apoptosis in brain cell death due to global ischemia, and reported that the expression of Bcl–2 mRNA increased at the time lapse of 2–30 hours after ischemia, maintaining the increased state up to 8 hours and at the time lapse of 24 hours, restoring the level of control group (26). In this paper, the change of BAX in hippocampus appeared decreasing in GV20–EA group and LI4–EA group at the time lapse of 9 hours after ischemia generation. This result is similar to that of, Kim (27) and Kim (28), which identified the decrease of BAX expression caused by EA application (29). In addition, Li et al. also reported BAX expression decrease by EA application. In rat model conducting reperfusion after middle cerebral artery occlusion (MCAO), they applied EA (3 Hz, 1–3 mA, continuous waves) to GV14 and GV20 for 30 minutes and observed the BAX change. They found that BAX decrease appeared statistically significant (p<.05) in EA–applied groups. Furthermore, in this paper, BAX expression in EA–applied groups increased more at the time lapse of 12 hours than 9 hours, and this is considered to be a delayed effect of EA application on BAX expression (30, 31).
In 1986, nuclear factor kappa B (NF-κB) was found as a transcription factor required to make immunoglobulin kappa chain in B cell(32), and after that time it is revealed by numerous studies that NF-κB also plays an important role in immune responses to inflammation and so forth. However, based on the experimental evidence that transgenic mice deprived of p65, the chief components of NF-κB, died from the necrosis of hepatocyte in the stage of generation, and apoptosis increased by undestroyed Mutant 1kB, NF-κB was found to play an anti-apoptotic role, and therefore its importance was still more emphasized(33). NF-κB is thought to be activated by various kinds of ischemic stimulations and play an important role in tissue inflammation at the time of ischemia, and the increased activity of NF-κB at the time of ischemia stimulation actually has an inhibitory effect on cell deaths(33, 34). Moreover, NF-κB combines with the promotor area regulating the expression of interleukin(IL)-1β which is a pro-inflammatory cytokine, IL-6 and tumor necrosis factor, TNF-α and plays the key role in regulating their transcriptions. In this paper, NF-κB expression showed statistically significant decrease(p<.05) in non–EA group, GV20–EA group and L14–EA group, but in GV20 EA group and L14 EA group NF–κB expression also showed the phase of slightly increase. This result is similar to that of zhang et al, in which they observed the changes of COX–2 and NF–κB at the time lapse of 24, 48 and 72 hours respectively after re-perfusion of the SD rats with reversible middle cerebral artery occlusion(MCAO), and in GV20 EA group and GB7 EA group, found the statistically significant decrease(p<.05) of COX–2, which is inflammation accelerating factor(35). Therefore, EA application at the early stage of global ischemia is considered to regulate the expression of NF–κB and thus affect the expression of inflammation accelerating factors.

CONCLUSION

This study aimed to examine how EA application after transient global ischemia affects BAX and NF–κB in hippocampus, and observed the change of BAX and NF–κB in hippocampus tissues in GV20–EA group, L14–EA group and non–EA group at the time lapse of 6, 9 and 12 hours respectively, using Western blotting, and obtained the following results.

The expression of BAX was observed to decrease, though not statistically significant(p>.05), in GV20 EA group and L14 EA group at the time lapse of 9 hours after transient ischemia.

The expression of NF–κB showed the statistically significant(p<.05) decrease in GV20 EA group and L14 EA group, but non–EA group did not.

In the light of these results, it is thought that this study can be a basic data useful to the studies on therapy to reduce apoptosis and cell death by inflammation in hippocampus damage due to ischemia.

However, since this paper focuses on the early-phase apoptosis and inflammatory cell death in hippocampus of the white rat, it seems necessary to study further how these factors are influenced from some other cell–death–related mechanisms after the longer time lapse. In addition, the further study on the changing trends of other factors related with apoptosis and inflammation seems also needed.

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