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Original Article

The Effects of Electro-Acupuncture the Rat with Induced MCAO

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Objectives : This study was aimed at examining the effects of the application of EA (electroacupuncture) at GV20 and LI4 in the early cerebral ischemia on the size of cerebral infarction, COX-2 and IL-6.
Methods : For this experiment, 21, six-week-old male S-D (Sprague - Dawley) rats weighting 160g to 200g were selected and randomly classified into 3 groups, seven rats in each group. Brain ischemia was simulated using a modified Koizumi method which was performed on each rat. In the GV20 group, the GV20 of the SD rats was stimulated for thirty minutes with acupunctural electrode low frequency stimulator five hours after inducement of ischemia. For the LI4 group, the LI4 was stimulated as above, while for the Ischemia group, no stimulation was applied. Twenty-four hours after the experiment, stained cerebral tissues were examined and an immuno-histological test was done to examine inflammatory reaction
Results : Out of the three groups, the LI4 group showed the smallest size of cerebral infarction and the Ischemia group showed the highest COX-2 (cyclooxygenase-2) expression value in the cortex of the cerebrum. In addition, the LI4 group showed the lowest COX-2 expression value in unknown putamen out of the three groups.
Conclusions : We infer that EA, applied at LI4 and GV20 in early ischemia, is effective in delaying the expression of IL-6 (interleukin-6) and COX-2, the inflammatory agents manifested from stroke. In addition, application at LI4, rather than GV20, can lower the expression value of the inflammatory agents. Further, EA can be an effective way to block early inflammatory reaction in stroke.

Key Words : MCAO, COX-2, IL-6, electro-acupuncture

Introduction

Stroke is a non-traumatic brain injury caused by cerebral infarction or hemorrhage. It causes disappearance of motor control, disturbance of sensation, damage to cognitive function or language function, posture imbalance and coma. Most patients have after-effects and have difficulty in being restored fully[1)]. Modalities of treatment in stroke rehabilitation in western medicine are physiotherapy, occupational therapy, and speech therapy, in addition to skilled medical and nursing care[2)]. These therapy methods

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are also used for such disorders in Korea, but medical acupuncture of Korean traditional medicine is also used in new therapy methods as alternative medicine[3].

Acupuncture is an important field of oriental medicine, and most diseases have been treated with it in Korea and China for several thousand years. While the effect of acupuncture is fully accepted, the WHO in 1979 listed 47 disorders as affected by acupuncture including pain control, alcoholism, smoking, drug abuse, cerebral infarction after discharge of bronchitis, and rehabilitation therapy for CNS injury from cerebral infarction[4]. A study using CBF SPECT reported[5] increase of blood flow of normal or stroke patient using acupuncture while another research showed cerebrovascular expansion causing increase of blood flow in brain tissue resulting oxygen saturation in brain cells[6,7]. In addition, Cho ZH *et al.*[8] reported acupoint stimuli effectiveness through stimulation of brain area of the related body site or internal organs in need of therapy.

Electro-acupuncture delivers electrical stimulation to the acupoints through the acupuncture needles. In particular, EA has been recommended as a complementary therapy for pain relief[9,10] and stroke rehabilitation in both Asian and western countries[11,12]. A considerable number of studies have been conducted investigating the effectiveness of EA therapy on patients with cerebral ischemia. Several beneficial outcomes were observed, such as reduced paralysis by increasing muscle strength, improved speech ability, reduced mental retardation, restored cerebral blood flow and improved locomotion[13,14,15].

It is known that IL-6 contributes to an excessive metabolism and hyperdioxide action in various known cytokines in acute stages of seriously brain-damaged patients. In other words, outgrowth is considered to be cerebral astrocytes by microglia, and IL-6 is known to cause inflammation, brain edema of activation, canal outgrowth, astrocytes hyperplasia and angiogenesis, endodermis function of collagen and recovery while performing an important function after brain damage[16]. Though the process of regeneration is contributed to by stimulation, IL- 6 gives direct influence to nerve growth factor of the central nervous system, and the extent to which brain damage is predicted is recognized as an important factor[17]. Recent studies which have attempted to measure IL-6 in neural cells after brain damage could be used to measure the degree of brain damage and clinically efficient brain injury factor. Shin[18] reported that mean value of serum of IL-6 measured in nerve cells after adult traumatic brain injury within 1 day increased significantly, 20 times more than normal.

Study of COX-generating fat mediators having inflammatory reaction is active recently, and progressing[19]. In the early 90s, COX was separated into two forms: COX-1 has the function to keep physiological homeostasis in tissues like stomach, kidney, plaque and blood vessels while COX-2 forms prostaglandin that mediates inflammatory reaction with increase in expression mainly around the area of inflammation[20]. Substances inducing expression of COX-2 include growth factor and tumor promoter as well as inflammatory cytokines such as IL-1β, TNF-α and IL-6[21].

In this study, based on the fact that the changes of COX-2 and IL-6 in brain damage in its early stages indicate recovery of brain damage, our research showed the effect on the change of COX-2 and IL-6 by stimulating acupoints related to the areas of damage using EA.

Material and Methods

1. Materials

21 healthy male Sprague-Dawley rats of six weeks ages and weight between 160 and 200g were used. They were divided 7-each into the GV20 group (GV20 EA low frequency), LI4 group (LI4 EA low frequency) and Ischemia group (ischemia only).

Temperature was maintained at 22℃, air humidity 45-55 %.

2. Method

A. Induction of ischemia

Brain ischemia was caused using a modified Koizumi method[22] performed on each group. Partial incision in the middle of the neck of the supine rat was made, and the left common carotid artery was opened. After separating the external carotid artery and internal carotid artery at the proximal part along the common carotid artery, the common carotid artery and external carotid artery were clipped and the blunt end of 5.0 monofilament nylon thread was pushed in to the branch of the common carotid artery, the middle cerebral artery was closed and sutured. All three groups were treated with the same method.

B. Experiment groups and control group

1. GV20 group (n = 7): A site on the crown of the head of the SD rat equivalent to the GV20 site was chosen, and stimulated five hours after ischemia elicitation.
2. LI4 group (n = 7): A forefoot site of the SD rat equivalent to the LI4 site was chosen, and stimulated five hours after ischemia elicitation.
3. Ischemia group (n = 7): EA was not given after ischemia elicitation.

For each acupuncture stimulation group, surgical tape was used to restrain the body, a site equivalent to the human acupoint was chosen, and EA stimulation was given using low frequency (PG6, ITO, JAPAN, 9 V, alternating current, 2 Hz).

C. Immunohistochemistry method

Rats were transcardially perfused with 250 ml 0.1 M PBS, pH 7.4, with heparin (1000 U/I) for 20 min followed by 500 ml of ice-cold fixative containing 4% paraformaldehyde in 0.1 M PBS, pH 7.4 for 30 min (n=3 rats per group). After postfixing for an additional 12 h in the same fixative at 4℃ condition, the brains were cryoprotected in 30 % sucrose in 0.1 M PBS, pH 7.4, for 48 h at 4℃ until sectioned. Coronal 40 μm-thick (Bregma: -3.14mm) sections obtained using a Leica 1800 cryostat (Leica, Germany) were collected in 0.1 M PBS, pH 7.4, and stored at 4℃ until processed. Free-floating sections were used for immunohistochemistry of COX-2 and IL-6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described below. Briefly, all treatments were carried out at room temperature except for the primary antibodies. Sections were blocked for endogenous peroxidase activity in 1% H_2O_2 in 0.1 M PBS, pH 7.4, for 30 min, then incubated for 30 min with 0.1 M PBS + 5% normal goat serum to reduce nonspecific staining. After that, sections were treated for 72 h at 4℃ with primary antibody solutions including anti-COX2 and anti-IL-6 (dilution 1:100 in 0.1 M PBS). Sections were immunostained using a standard biotin-avidin detection system (Vectastain ABC kit).

3. Data analysis

Changes in number of COX-2 and IL-6 were processed statistically using statistics package SAS. Changes in number of COX-2 and IL-6 between groups during the same period were processed with one-way ANOVA and one-way MANOVA.

Results

This study was aimed at examining the effects of the application of EA at GV20 and LI4 in the early cerebral ischemia on the size of cerebral infarction, COX-2 and IL-6. As for the test method, immunohistological measure was carried out to observe inflammatory reaction and TTC staining for observing the infarct volume specifically. Infarct was compared by naked eye for changes in volume. Number of expression was recognized based on 1 field under optical microscope for comparing the substance showing inflammatory reaction with COX-2 and IL-6.

1. Morphological change induced

A. 2,3,5-Triphenyltetrazolium (TTC) stain

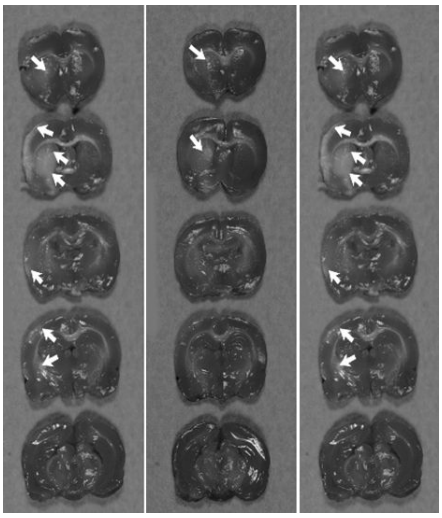

Fig. 1. Volume of cerebral infarction after ischemia. TTC staining indicated that the LI4 group was observed with the smallest size of cerebral infarction (A: GV20 group, B: LI4 group, C: Ischemia group)

B. Immunohistochemistry method

a. Cyclooxygenase-2 (COX-2)

Rats were divided into a GV20 group, an LI4 group, and an ischemia-only group, and statistical process was done, and extent of COX-2 expression numerical values was compared with cerebral cortex part and in caudate putamen part 24 hours after ischemia. Numeric value of COX-2 decreased depending on acupoint and application of EA in each group (table 1), (picture 3). In the cerebral cortex of each group, numeric value of COX-2 expression was 133.8 ± 13.1 in the LI4 group, 198.6 ± 14.4 in the GV20 group, and highest at 391.5 ± 10.1 in the Ischemia group. Caudate putamen recorded the lowest value of 261.3 ± 11 in the LI4 group.

In addition, since each P-value is considerably lower than significance level of 0.05 in statistical analysis, it was found that there were significant differences between the numeric values of COX-2 expression in the LI4, GV20 and Ischemia groups.

b. Interleukin-6(IL-6)

Extent of IL-6 expression numerical value was compared with cerebral cortex part in the caudate putamen area 24 hours after ischemia. That application right or wrong and acupoint of EA are obeyed in each group, and numerical value of IL-6 decreases (table 2) (picture 5) The IL-6 expression numerical

Table 1. COX-2 reaction numerical value change of each group

	Cerebral cortex	Caudate putamen
Ischemia group *	391.5±10.1	548.6±10
LI4 group†	133.8±13.1	261.3±11
GV20 group‡	198.6±14.4	347.0±9.2

(Mean ± SD)
* Ischemia group: no EA applied
† GV20 group: EA applied to GV20 acupoint
‡ LI4 group: EA applied to LI4 acupoint

Table 2. IL-6 reaction numerical value change of each group

	Cerebral cortex	Caudate putamen
Ischemia group*	382.3±10.4	429.5±11.1
LI4 group†	224.5±9.4	320.0±12.0
GV20 group‡	552.3±20.8	569.5±8.0

(Mean ± SD)
* Ischemia group: no EA applied
† GV20 group: EA applied to GV20 acupoint
‡ LI4 group: EA applied to LI4 acupoint

value taken in the brain cortex part of each group was 224.5 ±9.4 in the LI4 group, 552.3 ± 20.8 in the Ischemia group, and lowest at 382.3± 10.4 in the GV20 group. 382.3 ±10.4, which was the lowest numerical value, was discovered in the LI4 group, and it appeared even in the caudate putamen part in the GV20 and Ischemia group sequences. In addition, because each P-value was smaller than the level of significance 0.05 in statistical analysis in large measure in each GV20, LI4 and Ischemia group, it appeared that there was significant difference in the IL-6 expression numerical value.

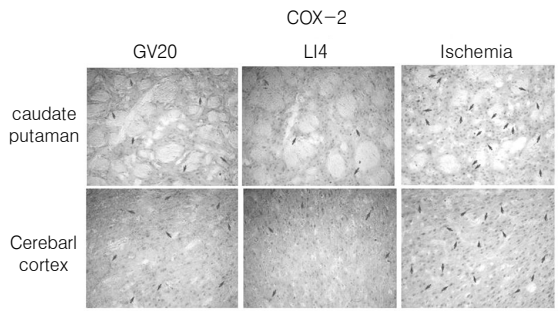

Fig. 2. Immunohistochemistry finding of COX-2. The LI4 group showed the lowest COX-2 expression value in caudate putamen; GV20 = EA applied at GV 20 acupoint, LI4 = EA applied at LI4 acupoint, Ischemia = no EA applied. (X200)

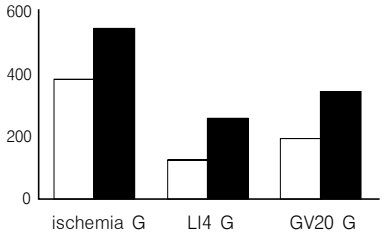

Fig. 3. COX-2 reaction numerical value change of each group

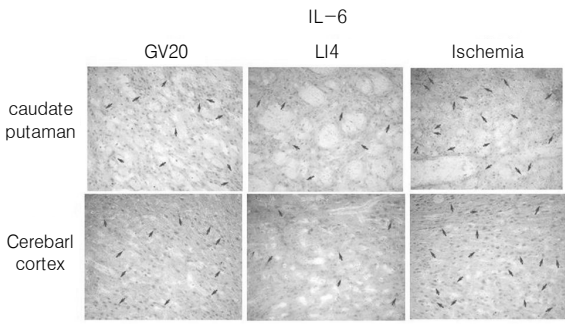

Fig. 4. Immunohistochemistry finding of IL-6

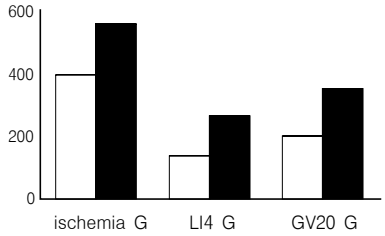

Fig. 5. IL-6 reaction numerical value change of each group

Discussion

Mechanisms for treatment of brain damage are not yet definitely known. It is a complicated problem, and it is difficult to analyze the influence of the inflammatory reaction on brain damage.

As for the COX known for prostaglandin H2 synthase, a catalyst has composition of prostanoids (prostaglandins and thromboxanes) from arachidonic acid. Two COX isoforms have been identified: COX-1 and COX-2[23]. The COX-1 gene has structural characteristics of a "housekeeping" gene and is mainly expressed constitutively. The COX-1 enzyme is relatively ubiquitous in the body and is the only isoform present in platelets, where it converts arachidonic acid to TXA2[24]. Expression of COX-2 is increased during inflammation and cellular transformation. Besides these, there are many things that induce COX-2 expression such as ischemia, trauma and hemorrhage. In addition, guidance is considered to be mitogen, endotoxin by reaction of cytokines. COX-2 is considered to rapidly follow tissue inflammation, and the reactants bring much cause of cytotoxic antibody consequences of inflammation[23].

Increase of COX-2 expression happens after inflammatory stimulation including ischemia and hypoxia. Outgrowth was able to be left by middle cerebral artery occlusion (MCAO) and was increased by brain ischemia, and S. Nogawa *et al.*[25] observed higher expression of COX-2 messenger RNA (mRNA).

H. Kinouchi *et al.*[26] said that six hours after ischemia, expression of COX-2 rose and it reached an absolute maximum between 12 - 24 hours and stayed higher until 48 hours.

In this study of expression of IL-6 at the cerebral cortex, it was observed to be 133.8 ± 13.1 in the LI4 group, 198.6 ± 14.4 in the GV20 group, and 391.5 ± 10.1 in the ischemia group. In the GV20 and LI4 groups, it decreased more than in the ischemia group. In addition, in the caudate putamen area, the LI4 group showed the lowest number at 261.3 ± 11. The GV20 group recorded 347.0 ± 9.2, which was more decreased than the ischemia group with 548.6 ± 10 and was similar to necrosis(or apoptosis) area of cells shown through TTC stain. In this case, EA stimulation at LI4 and GV20 prevents expression of COX-2 which is an inflammatory factor.

IL-6 is a known pleiotropic cytokine contributing to immunoreactive pacing, and is formed in various cells by anoxia and stimulation of ischemia[27]. Many inflammatory reactions are known with it being regulated by interleukins[28]. As for interleukins' effect on inflammation, it happens through outgrowth of diverse cells, including microglial cells, astrocytes, and leukocytes, is direct, and these regulate the central nervous system, apoptosis, differentiation, and proliferation. As for cytokines, there are associations for activity and supplement of leukocytes in the whole CNS. Furthermore, leukocyte activity and leukocyte stand, and IL-1 β, TNF-α, and IL-6 show expression increase of oil adhesion receptors of endothelial cells and astrocytes[29-32]. Some suggest[33] that IL-6 has more anti-inflammatory and immunosuppressive effects than proinflammatory cytokine while others report that activation of IL-6 induces expression of COX-2 and inflammatory factors[34,35]. In addition, the regeneration process is contributed to by stimulation[17], and IL-6 is generated in astrocytes, microglia, and brain endothelial cells after stimulation when direct influence is given to nerve growth factor in central nervous system during extravasation of blood[36]. Change of IL-6 appears in brain tissue, corresponding to infarct volume in cerebral ischemia damage, which means that it is also related to range of brain damage by hypoxia . IL-6 mRNA is reported to be up-regulated after ischemia of CNS.[18]

With regard to expression of IL-6 at the cerebral cortex, it was observed to be 224.5 ± 9.4 in the LI4 group, 552.3± 20.8 in the GV20 group, and 382.3 ± 10.4 in the Ischemia group. In the LI4 group, it decreased greatly but it rather increased in the GV20 group.

In addition, on the area of caudate putamen, the

lowest figure of 320.0 ± 12.0 was observed in the LI4 group, compared to 569.5 ± 8.0 in the GV20 group, and decreased more in the LI4 group than in the Ischemia group with 429.5 ± 11.1. This result shows that application of EA at LI4 resulted in prevention effects of inflammation with regard to expression of IL-6, the factor related to inflammation

However, in the GV20 group, expression of IL-6 increased, which means that expression of initial NF-κB during the progress of inflammation increases expression of IL-1β, IL-6 and TNF-α , and they are known to exist in the continuous line of increasing the expression of COX-2[34,35].

What is important in this process of cell death and inflammation is the relationship between degree of expression and time of expression of such factors. Since expression of IL-6 is the reaction that appears before expression of COX-2 among the results from this study, expression of more IL-6 made us expect expression of COX-2 in advance. That is, increase in COX-2 and rather low expression of IL-6 were found in the Ischemia group, which is considered as a point of time when expression of IL-6 decreases since cells which had already secreted IL-6 are ones that expressed COX-2. Results in the GV20 group with greater expression of IL-6 showed that expression of COX-2 is delayed as the progress of inflammation occurs more slowly than the expression of IL-6 that appeared in the Ischemia group. Results in the LI4 group were less expression of IL-6 and COX-2, which means that the progress stage of inflammation is slower than in the GV20 group. Such study results demonstrate that application of EA in the LI4 and GV20 areas has effects of delaying expression of IL-6 and COX-2, inflammatory factors expressed in stroke, and EA can be utilized for prevention of initial inflammation reaction of stroke.

Conclusion and Proposal

This study aimed at examining the effects of EA applied at GV20 and LI4 in early cerebral ischemia on the size of cerebral infarction, COX-2 and IL-6. For this, a total of twenty-one, six-week-old male SD rats weighing 160g to 200g were selected and randomly classified into three groups, seven in each.

All experimental animals were fixed. For the GV20 group, the bregma, corresponding to the acupoint GV20 of the SD rat, was stimulated for thirty minutes with acupunctural electrode low frequency stimulator (PG6, ITO, JAPAN, 9V, 2Hz) five hours after ischemia was induced; for the LI4 group, the acupoint LI4 was stimulated. For the ischemia group, no EA stimulation was applied.

Twenty-four hours after experiment, for the cerebral tissues of the experiment and ischemia groups, TTC staining was conducted for examining the size of the cerebral infarction and immunohistological testing was conducted to specially examine any inflammatory reaction.

1. The results from TTC staining showed the smallest size of cerebral infarction in the LI4 group.
2. The results from the immunohistological test indicated that the Ischemia group showed the highest COX-2 expression value in the cortex of the cerebrum, but according to the size of cerebral infarction it was the GV20 group.
3. The LI4 group showed the lowest COX-2 expression value in caudate putamen.
4. As for the expression value of IL-6, correlated with that of COX-2, the Ischemia group showed the highest expression value in the cortex of the cerebrum.
5. The LI4 group, with the smallest infarction, showed the lowest expression value in unknown putamen.

It can be inferred from the above results that EA, applied at LI4 and GV20 in early ischemia, has effects that can delay expression of IL-6 and COX-2, the inflammatory agents expressed from stroke. In addition, application at LI4, lowers expression value of the inflammatory agents more than at GV20. Further, EA can be an effective way to block early inflammatory reaction in stroke.

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