The Effect of *Acori Graminei Rhizoma* Pharmacopuncture at GV20 on Dementia in a Focal Cerebral Ischemia Mice Model

Yeo jin Jang¹, Min Kyung Kwak¹, Sang Jun Jeong¹, Hye Hwa Kim², Tae Gwang Kim³ and Jae Hong Kim¹,*

¹Department of Acupuncture & Moxibustion Medicine, College of Korean Medicine, Dong–Shin University
²Department of Ophthalmology, Otolaryngology & Dermatology Medicine, College of Korean Medicine, Dong–Shin University
³Department of Rehabilitation Medicine, College of Korean Medicine, Dong–Shin University

[Abstract]

Objectives: The purpose of this study was to examine the effects of *Acori Graminei Rhizoma* Pharmacopuncture (PA–AG) at GV20 on cerebral ischemia–induced dementia in Mice.

Methods: Mice were divided into the five following groups: normal, control, acupuncture, PA–AG (17 mg/kg), and PA–AG (34 mg/kg). All groups, except the normal group, had cerebral ischemia induced by occlusion of middle cerebral artery. The control group was not treated. The acupuncture, PA–AG (17 mg/kg), and PA–GA (34 mg/kg) groups were treated every other day with a total of 6 treatments. The effect of treatment was observed by Bax, Bcl-2, Bax/Bcl-2 ratio, cytochrome c, cresyl violet, and choline acetyltransferase staining.

Results: In the PA–AG (34 mg/kg) group, the intensity of Bax was decreased and the intensity of Bcl–2 was increased. The Bax/Bcl–2 ratio also decreased in the PA–AG (34 mg/kg) group. The intensity of cytochrome c protein stain was decreased in the PA–AG (17 mg/kg) group. The density of neurons stained by cresyl violet and choline acetyltransferase (ChAT) was increased in the AT, PA–AG (17 mg/kg), and PA–AG (34 mg/kg) groups when compared with that of the control group.

Conclusion: PA–AG at GV20 was effective on cerebral ischemia–induced dementia in mice.

Key words: *Acori Graminei Rhizoma* Pharmacopuncture; GV20; Dementia


※ "This study was supported by the Convergence of Conventional Medicine and Traditional Korean Medicine R&D program funded by the Ministry of Health & Welfare through the Korea Health Industry Development Institute(KHIDI)(HI14C0862)

✱ Corresponding author: Department of Acupuncture & Moxibustion Medicine, Gwangju Korean Medicine Hospital of Dong-Shin University, 141, Wolsan-ro, Nam–gu, Gwangju, 503–232, Republic of Korea

Tel : +82 –62–350–7209 E–mail : nahongai@hanmail.net
I. Introduction

Dementia is a disease that causes physical and mental disability in a patient, significantly lowering the quality of life. According to the Ministry of Health and Welfare, the number of patients with dementia nationwide is estimated to be 610,000 in 2014. In the age of aging, the number of patients with dementia is increasing exponentially and is estimated to reach 1 million in 2024 and 2 million in 2041. The direct and indirect costs of dementia management are enormous and the Ministry of Health and Welfare estimates that 13 trillion KRW was spent in 2014 and the cost will double every 10 years.

In Oriental medicine, dementia has been treated in conditions such as depressive–manic psychosis, forgetfulness, depression, phlegm–dampness syndrome, and consumptive disease. The causes are mainly liver–kidney essential qi insufficiency, spleen–kidney depletion syndrome, syndrome of phlegm turbidity obstruction orifices, blood stasis due to qi stagnation, intense heat toxin, and qi–blood deficiency syndrome. The cures are to mainly tonify qi and replenish the blood, tonify the kidney and fortify the spleen, clear phlegm and resolve turbidity, open the orifices and clear phlegm, and boost qi and nourish the blood.

Acorus Gramineus opens the orifices and tranquillizes medicinal (開竅安神藥). It transforms into dampness and increases the appetite, opens the orifices and clears phlegm, blooms the spirit and promotes mental health effects, and is clinically used for phlegm–dampness clouding, syncope, mental confusion, depressive–manic psychosis, dementia, and forgetfulness due to a dysfunction of brain and heart.

GV20 clear conscious, restore yang and security collapse (淸神志, 恢復陽和固脫), elevates a sunken yang–qi (陽陽氣下陷), clears heat and open the orifices (清熱開竅) to mainly treats headache, wind stroke (中風), lockjaw (口噤), forgetfulness, prolapse (脫肛), nasal blockage (鼻塞), dizziness (目眩).

So far, studies on dementia have been conducted on herbal medicine treatments such as the enhancing effects of Gwibi–tang on cognitive function and memory in a scopolamine–induced dementia rat model and monophosphoremic agents. Studies on specific acupuncture point stimulation using a single drug pharmacopuncture solution have not been studied sufficiently.

The authors review the effectiveness of PA–AG at GV20. The expression of Bax, Bcl–2 and cytochrome c, which are related to neuronal cytotoxicity and death, was analyzed in the hippocampus. We observed Cresyl violet staining that has a neuroprotective effect and ChAT expression related to the neurotransmitter receptor.

II. Materials and Methods

1. Materials

1) Animals

Male Sprague Dawley rats weighing about 210–230 g were fed with solid feed (pellet, Samyang Corporation, Korea) and water, and housed in a kennel (temperature 24 ± 1 ℃, humidity 60 ± 5%). After the adaptation to the laboratory environment for more than one week, it was used in the experiment. During the experiment, water and solid feed were also freely consumed (No. 2015–09–01).

2) Preparation of pharmacopuncture solution

200 g of Acorus gramineus soland purchased from Dongshin University Hospital was boiled with 1,000 ml of distilled water for 3 hours and centrifuged at 5,000 rpm for 30 minutes with a centrifuge (Centricon T–42K, Italy). The supernatant
was collected and concentrated under reduced pressure to 100 ml by evaporating the water with a rotary evaporator (Buchi, Netherlands). The concentrated drug solution was lyophilized at $-70 \, ^\circ\text{C}$ with a freeze dryer (Samwon Co., Korea) and the final amount of the drug solution was 12.11 g. These were diluted with physiological saline and adjusted to pH 7 with a pH meter (ORION, U.S.A) and stored in the refrigerator.

3) Injection of the pharmacopuncture solution

The pharmacopuncture solution was injected using an insulin syringe (31 G × 8 mm, BD, USA).

2. Methods

1) Ischemic local brain injury caused by occlusion

Focal cerebral ischemia was caused by the occlusion of the left middle cerebral artery according to the method of Zea Longa et al. Mice were induced by inhalation anesthesia with 80% O$_2$ gas using 5% isoflurane (Hana Pharm, Korea) and maintained with 2% isoflurane. In order to obstruct the left middle cerebral artery, the skin was cut along the midline of the neck, and the left common carotid artery was exposed between the sternohyoideus and sternal masticatory muscle to secure the distal portion of the left internal carotid artery. After ligation using a microvascular clip (WPI, USA), which was ligated with a 6–0 silk suture thread with the left common carotid artery and the left proximal outer carotid bifurcation to prevent bleeding between the internal carotid artery and the left intraluminal filament. The vessels were dissected using microvascular scissors (WPI, U.S.A.) at the distal part of the internal carotid artery, which was 1 cm in the left external carotid artery and internal carotid artery branch. After removal of the microvascular clips, an intraluminal filament (diameter 0.28 mm rounded tip) with a 20-mm dental impression agent (Durelon, Germany) was inserted into the left internal carotid artery until the intraluminal filament was felt in the left internal carotid artery (about 17 mm) and was gently inserted into the left middle cerebral artery, resulting in focal ischemia of the left middle cerebral artery. To prevent bleeding, the intraluminal filament insertion site at the left internal carotid artery was lightly tied and the incised skin was sutured.

2) Group separation and treatment

The subjects were divided into five groups: normal (n = 5), ischemia-induced control (Control, n = 5), and those treated with either acupuncture at GV20 (AT, n = 5), pharmacopuncture (17 mg/kg) at GV20 PA−AG [17 mg/kg], n=5), or pharmacopuncture (34 mg/kg) at GV20 PA−AG (34 mg/kg), n=5) after the induction of ischemia. Treatment was performed twice a day with 20 μl of GV20 for a total of 6 sessions.

3. Western blotting

1) Protein preparation

The excised brain area was rapidly frozen in liquid nitrogen and stored at $-70 \, ^\circ\text{C}$ until analysis. Bax, Bcl−2, and Cytochrome c protein expression were observed by Western blot. The brain tissue (300 mg) of mice were homogenized by adding 500 μl of NP40 lysis buffer (pH 8.0, 50 mM Tris HCl; 150 mM NaCl; 5 mM EDTA; 0.2 mM phenylmethylsulfonylfluoride [PMSF]; 1% NP−40; 1 mM benzamidine; 1 μg/ml trypsin inhibitor) and a 1X Protease Cocktail inhibitor (BD, U.S.A.) mixture. The sample was placed on ice for 20 minutes, centrifuged at 12,000 rpm for 20 minutes at 4 °C, and the supernatant was separated. This sample was quantitated using a bicinchoninic acid (BCA) assay kit (Pierce, USA). BCA solution (100 μl; A: B = 50:1) and 5 μl of protein were placed in a 96–well plate, incubated at 37 °C for 20 minutes, and then measured by at 570 nm using an ELISA reader (Bio−Rad, USA).
2) Electrophoresis

Polyacrylamide resolving gel (12%) was polymerized for 30 minutes, then a 5% polyacrylamide stacking gel was poured on top of the resolving gel, plugged with a comb, and allowed to solidify for 30 minutes.

The gel was installed on a running tank and running buffer (0.025 M Tris, 0.192 M glycine, 10% SDS) at pH 8.3 was poured into the tank. The protein sample was added to 5 μl of a 5X SDS sample buffer (pH 6.8, 100 mM Tris, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 200 mM DTT, 10% 2-mercaptoethanol) and boiled at 100 ℃ for 5 minutes. The stacking gel was electrophoresed at 80 V and the resolving gel was electrophoresed at 130 V.

3) Electroblotting

After the electrophoresis, the filter paper and nitrocellulose membrane were placed in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) and transferred at 20 mA for 24 hours. The membrane was blocked with 5% nonfat milk and TBST (0.1% Tween 20 in a pH 7.4 Tris-based saline buffer) for 1 hour and washed twice with TBST.

Bax antibody (1:1500, Cell Signaling, U.S.A.), Bcl-2 antibody (1:1500, Santa Cruz Biotechnologies, U.S.A.), Cytochrome c antibody (1:2000, Becton Dickinson, U.S.A.), and β-actin antibody (1:2000, Santa Cruz Biotechnologies, U.S.A.) were diluted in TBST overnight at 4 ℃.

Bax and Bcl-2 were incubated with horseradish peroxidase-labeled anti-rabbit IgG (1:1500, Cell Signaling, USA) and cytochrome c with horseradish peroxidase-labeled anti-mouse IgG, β-actin was incubated with horseradish peroxidase-labeled anti-goat IgG (1:2000, Santa Cruz Biotechnologies, USA) for 1 hour and washed at 4 ℃ times with TBST.

4) Immunohistochemistry

1) Cresyl violet staining

Immediately after the completion of all the experiments, the mice were anesthetized with urethane (25%, Sigma, USA), perfused with 200 ml of 0.9% saline followed by 800 ml of 5% formalin solution (fixative). The first 200 ml of fixative was run at a fast flow rate for 2 minutes and the remaining 800 ml was run slowly. Mice that were fixed has their brains removed, fixed with 10% formalin solution, and stored at 4 ℃ in phosphate buffered saline (PBS) containing 30% sucrose for 1 day. The next day, the frozen brain was cut into 30-μm thick slices. The tissue was washed several times with PBS and transferred into the following solutions in the following order: xylene (5 min), 100% alcohol (2 min), 95% alcohol (1 min), 70% alcohol (1 min), and distilled water (2 min). After degreasing and dehydration, the cells were stained with cresyl violet buffer (5 min). The stained tissue was magnified at 40X using an optical microscope (Nikon, Japan). The density of neurons was measured using the Scion image program (Scion Corp., MD, USA).

2) Choline acetyltransferase (ChAT)

The brain tissue was initially washed with 0.1 M PBS three times, blocked with 2% rabbit serum for 30 minutes, and then used with ChAT anti-body (1:500, monoclonal, Millipore, U.S.A.). A 0.1% sodium azide (Sigma, St. Louis, Mo., USA) solution in 0.1 M PBS was used to make a 500-fold dilution of the primary antibody. Brain tissue was cultured in primary antiserum at 4 ℃ for 24 hours. The tissues were washed three times with 0.1 M PBS and reacted with biotinylated universal secondary antibody (Quick Kit: Vector Laboratories, Burlingame, CA, USA) for 30 min at 37 ℃. After washing three times with 0.1 M PBS and incubated with horseradish peroxidase-labeled universal secondary antibody (Quick Kit: Vector Laboratories, Burlingame, CA, USA) for 1 hour and washed at 4 ℃ times with TBST.

After removal of TBST, Lumiflash Infinity Chemiluminescent substrate (Visual protein, U.S.A.) was added to the membrane and allowed to react for 5 minutes. Bands of the expressed Bax, Bcl-2, Cytochrome c, and β-actin were developed in developer (1:5, Dongjin, Korea) and fixer (1:5, Dongjin, Korea) in ECL film (Amersham, U.K.).

The intensity of expressed Bax, Bcl-2, cytochrome c, and β-actin was measured using AlphaEase FC StandAlone Software (Alpha Innotech, USA).

http://dx.doi.org/10.13045/acupunct.2017088
times with 0.1 M PBS, the brain tissues were immersed in a preformed streptavidin peroxidase complex (Quick Kit; Vector Laboratories, Burlingame, CA, USA) for 30 min at 37 °C. After washing three times with 0.1 M PBS, the tissues were expressed using diaminobenzidine (DAB) as a colorant. The color development was stopped with 0.1 M phosphate buffer. After the slide was dehydrated, the stained tissue was magnified 40X using an optical microscope (Nikon, Japan) and the density of neurons was measured using the Scion image program (Scion Corp., MD, USA).

5. Statistics

All measurements are expressed as the average value and standard error (mean ± standard error) using the Excel statistic (Microsoft, USA). The statistical analysis of each group was conducted using the Mann–Whitney U test in a non-parametric manner using SPSS (IBM, USA).

The significance of each test group was tested at α = 0.05 level (p < 0.05) and α = 0.01 level (p < 0.01) compared to the control group.

III. Results

1. Effect on Bax level

Bax changes were observed at 171.9 ± 2.1 (*1000 OD) in the normal group, 184.9 ± 1 (*1000 OD) in the control group, 187.2 ± 1.5 (*1000 OD) in the AT group, 181.2 ± 1.8 (*1000 OD) in the PA–AG (17 mg/kg) group and 154.2 ± 2.2 (*1000 OD) in the PA–AG (34 mg/kg) group.

Compared to the normal group, the measurements of the control group were significantly higher. The levels in the PA–AG (34 mg/kg) group had significantly decreased when compared to that of the control group (Fig. 1).

2. Effect on Bcl–2 level

Bcl–2 level changes were observed at 183.1 ± 1.9 (*1000 OD) in the normal group, 173.3 ± 2.8 (*1000 OD) in the control group, 176.7 ± 2.9 (*1000 OD) in the AT group, 181.7 ± 2.3 (*1000 OD) in the PA–AG (17 mg/kg) group, and 183.3 ± 1.8 (*1000 OD) in the PA–AG (34 mg/kg) group.

Compared with the normal group, the measurements of the control group were significantly lower. The PA–AG (34 mg/kg) group was significantly increased when compared with that of the control group (Fig. 2).

3. Effect on the Bax/Bcl–2 ratio

Changes in the Bax/Bcl–2 ratio were observed at a measurement of 0.94 ± 0.01 in the normal group, 1.07 ± 0.01 in the control group, 1.06 ± 0.02 in the AT group, 1.00 ± 0.01 in the PA–AG (17 mg/kg) group and 0.84 ± 0.01 in the PA–AG (34 mg/kg) group.

Compared with the normal group, the measurements of the control group were significantly increased. The measurements of the PA–AG (34
mg/kg) and PA-AG (17 mg/kg) groups had significantly decreased when compared with that of the control group (Fig. 3).

4. Effect on Cytochrome C level

Changes in the levels of cytochrome C were observed at 137.2 ± 1.5 (*1000 OD) in the normal group, 199.7 ± 5.7 (*1000 OD) in the control group, 197.1 ± 5.6 (*1000 OD) in the AT group, 179.5 ± 2.5 (*1000 OD) in the PA-AG (17 mg/kg) group and 200.9 ± 5.3 (*1000 OD) in the PA-AG (34 mg/kg) group. Compared to the normal group, the measurement of the control group was significantly higher. The PA-AG (17 mg/kg) group was significantly decreased when compared to that of the control group (Fig. 4).

Fig. 2. Changes on the Bcl-2 level in the brain after acupuncture (AT) and phamacopuncture in a MCAO rat

Values are expressed Mean ± SE; MCAO, Middle Cerebral Artery Occlusion. AT, acupuncture treatment. PA-AG (17 mg/kg), phamacopuncture–Acoris Gramineus Solander 17 mg/kg. PA-AG (34 mg/kg), phamacopuncture–Acoris Gramineus Solander 34 mg/kg. * p<0.05 compared with normal. # p<0.05 compared with control.

Fig. 3. Changes of the Bax/Bcl-2 ratio level of brain after acupuncture (AT) and phamacopuncture in a MCAO rat

Values are expressed Mean ± SE; MCAO, Middle Cerebral Artery Occlusion. AT, acupuncture treatment. PA-AG (17 mg/kg), phamacopuncture–Acoris Gramineus Solander 17 mg/kg. PA-AG (34 mg/kg), phamacopuncture–Acoris Gramineus Solander 34 mg/kg. ## p<0.01 compared with normal. * p<0.05, ** p<0.01 compared with control.

Fig. 4. Changes on the cytochrome C level of brain after treatment according to acupuncture (AT), phamacopuncture in MCAO rat

Values are expressed Mean ± SE; MCAO, Middle Cerebral Artery Occlusion. AT, acupuncture treatment. PA-AG (17 mg/kg), phamacopuncture–Acoris Gramineus Solander 17 mg/kg. PA-AG (34 mg/kg), phamacopuncture–Acoris Gramineus Solander 34 mg/kg. ## p<0.01 compared with normal. * p<0.05 compared with control.

Fig. 5. Changes on the cresylviolet stained neural activity of hippocampus after acupuncture (AT) and phamacopuncture in a MCAO rat

Values are expressed Mean ± SE; MCAO, Middle Cerebral Artery Occlusion. AT, acupuncture treatment. PA-AG (17 mg/kg), phamacopuncture–Acoris Gramineus Solander 17 mg/kg. PA-AG (34 mg/kg), phamacopuncture–Acoris Gramineus Solander 34 mg/kg. ## p<0.01 compared with normal. * p<0.05, ** p<0.01 compared with the control.
5. The effect of Cresyl violet on stained neural activity

As a result of observing the impact of cresyl violet stained neural activity changes in the hippocampus, the normal group had an index of 22.8 ± 0.7 and the control group was 12.9 ± 0.7. The index of the AT group was 17.7 ± 1.0, the PA– AG (17 mg/kg) group was 15.4 ± 0.8, and the PA– AG (34 mg/kg) group was 18.2 ± 1.3.

Compared to the normal group, the control group was significantly lower. Compared to the control group, the AT, PA–AG (17 mg/kg), and PA–AG (34 mg/kg) groups were significantly increased (Figs. 5, 6).

Fig. 6. Effect of the Cresylviolet stained neural activity of hippocampus after acupuncture (AT) and phamacopuncture in a MCAO rat

A, normal; B, control; C, AT; D, PA–AG (17 mg/kg); E, PA–AG (34 mg/kg).

Values are expressed Mean ± SE; MCAO, Middle Cerebral Artery Occlusion. AT, acupuncture treatment. PA–AG (17 mg/kg), phamacopuncture–Acoris Gramineus Solander 17 mg/kg. PA–AG (34 mg/kg), phamacopuncture–Acoris Gramineus Solander 34 mg/kg.
6. ChAT stained neural activity

The observations of the impact of ChAT–stained neural activity changes in the hippocampus includes an index of 10.7 ± 1.1 in the normal group and 7.1 ± 0.8 in the control group. The index of the AT group was 9.1 ± 0.5, the PA–AG (17 mg/kg) group was 13.1 ± 0.6, and the PA–AG (34 mg/kg) group was 13.2 ± 0.6.

Compared with the normal group, the measurements in the control group were significantly lower. Compared with the control group, the measurements of the AT, PA–AG (17 mg/kg), and PA–AG (34 mg/kg) groups were significantly increased (Fig. 7, 8).

IV. Discussion

Dementia is a marked decline in intellectual abilities that have reached a certain level or were once acquired. It is usually chronic and progressive and is a highly cortical dysfunction, including memory, thinking ability, comprehension ability, computing ability, learning ability, language and judgment). The most common types of dementia are Alzheimer–type dementia, accounting for more than 50%, followed by vascular dementia accounting for more than 20%[10]. However, the fact that Alzheimer’s dementia is also caused by vascularity has surfaced and the importance of vascular dementia is being highlighted. Vascular dementia is the damage of nerve cells due to cerebrovascular lesions. The major part of the brain involved in memory, cognitive functioning, and behavioral regulation fails to function, causing symptoms such as memory loss, paralysis, and speech disturbances. Thus, clinical symptoms such as gait disturbances, movement disorders, postural instability, recurrent falls, frequency or urinary incontinence, changes in personality and emotions, and impaired performance may occur[13].

Although vascular dementia is not mentioned separately in oriental medicine, a recognition of mental disorder caused by cerebrovascular disorder can be found in the literature.

Acorus Gramineus is a tranquilizing medicine that opens the orifices. It involves a hot flavor, is warm nature, nontoxic, and goes to the meridian of the heart and stomach. It has transformed dampness, increases the appetite, opens the orifices, clears phlegm, blooms the spirit, promotes mental effects, and is clinically used for syncope, mental confusion, depressive–manic psychosis, dementia, forgetfulness due to dysfunctions of the brain and heart[9].

GV20 is the intersection of the governor, bladder meridian, gallbladder, and liver meridians and is a triple energizer. It is located at the intersection between the midline of the head and the line connecting the apaxes of both ears. It has clears the conscious, restores yang and security collapse, elevates a sunken yang–qi, is heat–clearing, and opens the orifices. It mainly treats headache, wind stroke (中風), lockjaw (口噤), forgetfulness, prolapse (脱肛), nasal blockage (鼻塞), and dizziness (目眩)[4,13]. Currently, it is applied to diseases such as stroke, cerebrovascular circulation disorder, migraine, vertigo, and forgetfulness[10].
To investigate the effects of PA-AG at GV20 on mice with dementia, the expression of Bax and Bcl-2, and cytochrome c, the Bax/Bcl-2 ratio, neural tissue density using Cresyl violet, and ChAT expression were examined.

Bax, which is mainly present in the cytoplasm, migrates to the outer membrane of the mitochondria during stress, such as DNA damage, and induces apoptosis by liberating cytochrome c via permeation of the mitochondria. The exact mechanism by which Bax causes this phenomenon has not yet been clarified, but is known to be caused by oligomerization of the Bax proteins in the mitochondrial membrane, Bcl-2 is a typical factor that inhibits the action of Bax. It inhibits the formation of the Bax oligomer in the mitochondrial membrane and inhibits the actions of Bax, which ultimately inhibits apoptosis and promotes cell survival. Therefore, it is known that the Bax/Bcl-2 ratio is important for apoptosis because Bcl-2 decreases

Fig. 8. Effect of the ChAT stained neural activity of hippocampus after acupuncture (AT) and phamacopuncture in a MCAO rat

A, normal; B, control; C, AT; D, PA-AG (17 mg/kg); E, PA-AG (34 mg/kg).

Values are expressed Mean ± SE; MCAO, Middle Cerebral Artery Occlusion. AT, acupuncture treatment. PA-AG (17 mg/kg), phamacopuncture–Acoris Gramineus Solander 17 mg/kg. PA-AG (34 mg/kg), phamacopuncture–Acoris Gramineus Solander 34 mg/kg.

http://dx.doi.org/10.13045/acupunct.2017088
and Bax increases when apoptosis is induced\textsuperscript{15}.

Through observing the changes in Bax in this experiment, it was found that the AT group had increased levels of Bax when compared to that of the control group, but the PA–AG (17 mg/kg) and PA–AG (34 mg/kg) groups had decreased levels. This indicates that PA–AG at GV20 can inhibit Bax, a factor associated with brain cell death. In addition, the PA–AG (34 mg/kg) group was markedly decreased.

Through observing the change in Bcl–2 to inhibit the action of the Bax, its levels were increased in the AT, PA–AG (17 mg/kg), and PA–AG (34 mg/kg) groups when compared to that of the control. This suggests that acupuncture at GV20 may be involved in the anti-apoptotic action. The levels in the PA–AG (34 mg/kg) group were significantly increased, suggesting that PA–AG at GV20 contributes to the inhibition of the action of Bax.

The changes in the Bax/Bcl–2 ratio, which are important for apoptosis, were found to be decreased in the AT, PA–AG (17 mg/kg), and PA–AG (34 mg/kg) groups when compared to that of the control group. In particular, when viewed as significantly reduced in PA–AG (17 mg/kg) group and PA–AG (34 mg/kg) group, PA–AG at GV20 this is thought to be able to maximize the effect acupuncture at GV20.

Cytochrome c, which is a factor that promotes apoptosis, was observed to be decreased in all experimental groups when compared to that of the control group, indicating that acupuncture at GV20 was effective. However, PA–AG (17 mg/kg) group was significantly decreased. Therefore, further studies are needed to determine whether it is more effective at low concentrations or at high concentrations.

The density of neuronal cells in the hippocampus region was significantly increased in the AT, PA–AG (17 mg/kg), and PA–AG (34 mg/kg) groups when compared to that of the control group by Cresyl violet staining. Acupuncture and PA–AG at GV20 were found to be effective in protecting neuronal damage.

ChAT is a synthesizing enzyme of acetylcholine (ACh) which is a neurotransmitter. Reduction of ChAT activity leads to the reduction of ACh, resulting in deterioration of memory, learning ability, and concentration\textsuperscript{16}. In the present study, the effects of ChAT stained neural activity on the hippocampus were significantly increased in all experimental groups when compared to that of the control group, indicating that acupuncture and PA–AG at GV20 could activate neurotransmitter related functions.

In conclusion, PA–AG at GV20 has significant effects on cerebral ischemia–induced dementia. It is necessary to continue researching the optimal concentration and mechanisms of the application of this system for use in dementia induced by cerebral ischemia.

V. Conclusions

To investigate the effects of PA–AG at GV20, we analyzed the expression of Bax, Bcl–2, and cytochrome c, which are related to neuronal cytotoxicity and death in the hippocampus. We also observed a Cresyl violet neuroprotective effect and ChAT expression related to neurotransmitter receptors. The following is the summary of the report of this study:

1. Bax levels were significantly decreased in the PA–AG (34 mg/kg) group when compared to that of the control group.
2. Bcl–2 levels were significantly increased in the PA–AG (34 mg/kg) group when compared to that of the control group.
3. In regards to the Bax/Bcl–2 ratio, the PA–AG (17 mg/kg) and PA–AG (34 mg/kg) groups had significantly decreased levels when compared to that of the control group.
4. Cytochrome C levels were significantly decreased in the PA–AG (17 mg/kg) group when compared to that of the control group.
5. The effect of the hippocampus on Cresyl violet stained neural activity was significantly increased in the AT, PA-AG (17 mg/kg), and PA-AG (34 mg/kg) groups when compared to that of the control group.

6. As a result of observing the effect of hippocampus on ChAT stained neural activity, the AT, PA-AG (17 mg/kg), and PA-AG (34 mg/kg) groups had significantly increased levels of activity when compared to that of the control group.

VI. References
