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Effects of the Bee Venom Herbal Acupuncture on the Neurotransmitters of the Rat Brain Cortex

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ABSTRACT

In order to study the effects of bee venom herbal acupuncture on the neurotransmitters of the rat brain cortex, herbal acupuncture with the bee venom group and normal saline group was performed bilaterally on the point corresponding to LI 4 of the rat. The average optical density of the neurotransmitters from the cerebral cortex was analyzed 30 minutes after the herbal acupuncture with immunohistochemical method.

The results were as follows:

1. The density of NADPH-diaphorase in the bee venom group was increased significantly at the motor cortex, visual cortex, auditory cortex, cingulate cortex, retrosplenial cortex, and perirhinal cortex, compared to the normal saline group.

2. The average optical density of vasoactive intestinal peptide in the bee venom group had significant changes at the insular cortex, retrosplenial cortex, and perirhinal cortex, compared to the normal saline group.

3. The average optical density of neuropeptide-Y in the bee venom group increased

significantly at the visual cortex and cingulate cortex, compared to the normal saline group.

Key Words: bee venom, NADPH-diaphorase, vasoactive intestinal peptide, neuropeptide-Y

I . Introduction

Bee venom herbal acupuncture therapy[1] is a new type of treatment method to balance the bodily functions and improve the condition by biochemical effects. This is done by extracting the venom from venom follicle of the honey bee and injecting the processed venom into an area associated with specific illness.

In western medicine, the usage of bee venom for the treatment is recorded on papyrus as early as 2000 B.C. in Egypt and Babylonia. It has been used in the folk remedy for various pain control and inflammative diseases[1]. First clinical study was done by a Frechman Desjardins in 1858 on the usage of bee venom for rheumatoidal diseases, and in 1968, E. Haberman[36] published constituents of the bee venom and many experiments and clinical studies followed. First documentary record in terms of Oriental medicine on the bee venom was unearthed in 1973 in China as 2 cases of the bee venom therapy was recorded on the ancient text dating back to 168 B.C. In this document, the bee venom is gathered and processed before applying on the skin. 『Book of Regimen』 cites one way of the bee venom application: a chicken muscle treated with the bee venom is tempered with jujube oil and applied on a rag, and this rag is rubbed on the skin.

Another way is mentioned in the 『Book of Miscellaneous Diseases』 as the dog's liver processed with the bee venom is extracted using the vinegar as a solvent and the extract is applied onto the skin using a cotton pad[16].

Among the various types of bee, honey bee's (Apis mellifera ligustica) venom has characteristics of bitter and pungent taste, neutral temperature[17], and is known to tonify, pacify, stop cough, dispel wind-damp, alleviate pain,

anti-inflammation, increase immunity, anti-cancer, promote adrenal secretion, and restore lymphocytes and rbc[17,18].

Thus, the bee venom is widely used for treating myalgia, acute and chronic arthritis, hypertension, rheumatism, skin diseases, headache, lumbago, contusion[8], and etc.

Studies on the bee venom acupuncture had been mainly on anti-phlogistic, analgesic, immunity, and allergic reaction[7,9,15]. Partial researches on the central nervous system associated with pain control are made these days, but no research has been done on the overall changes in the brain region so far. This study was undertaken to evaluate the effects of the bee venom herbal acupuncture on the central nervous system. Bee venom was injected subcutaneously on the white rat on the point corresponding to LI 4[6] and changes of the neurotransmitters in the cerebral cortex was examined with immunohistochemical method and significant results were obtained.

Ⅱ. Experiment

1. Experiment Animals

White rat with the weight of approximately 200g were fed with solid pellet feed and plenty of water. The experiment was carried out after one week of adaptation to the laboratory environment.

2. Materials

1) Bee venom (Api-toxin): Bee venom collected using an extractor (electromagnetic stimulation using a microchip) was dried. Then 0.1g of the bee venom was diluted with 99.9ml of the distilled water to make 0.1% bee venom solution and used in the experiment.

2) Injection apparatus: Disposable injector (26 gauge needle, Green Cross Corp, Korea) of 1.0ml was used for both the bee venom and the saline solution.

3. Experiment Methods

1) Acupuncture point: Injected bilaterally on the point corresponding to LI 4 of the rat

2) Disposition method: Each of the saline solution injected groups and the bee venom groups was composed of 6 mice. Both groups were injected subcutaneously with 0.05ml of the saline

solution and the bee venom extract respectively, and tissue disposition was made after 30 minutes.

4. Tissue disposition

The rat was dissected after anesthetizing with pentobarbital sodium (60mg/kg) in the abdomen. Through the left ventricle, 0.05M of phosphate buffered saline (PBS) was injected for a minute and 4% paraformaldehyde dissolved in 0.1M phosphate buffer was circulated through for 10 minutes. Here, the circulation speed was set at 50~60ml/min. Then the brain was removed and sliced in the thickness of 4-6mm and stored in the fixative for 16-18 hours at 4℃. After this process, the brain slices were stored into 0.1M PBS added with 20% sucrose solution for 2 to 5 days. Using Cryocut (Leica, Germany), the slices went through successive cross cutting to yield thickness of 40㎛, and 1 out of 5 tissues was dyed.

5. Histochemistry of NADPH-diaphorase

Tissue slice was reacted in the mixed solution of 0.1% β-NADPH, 0.01% nitroblue tetrazolium, 0.3% Triton X-100 dissolved in 0.1M PB for 60-90 minutes at 37℃. When the desired reaction is yielded, tissue was washed with PBS to stop further coloration.

6. Immunohistochemistry of NPY and VIP

To deactivate the endogenous peroxidase, tissue slices were reacted in 1% H_2O_2 diluted with PBS for 15 minutes. Then they were washed thrice with PBS for 10 minutes and then shake cultured for 48 hours at 4℃ in the primary antibody solution of 0.05% bovine serum albumin, 1.5% Normal serum, and 0.3% Triton X-100 diluted with primary antibody of rabbit (or mouse). (Table 1). When the reaction is completed in the primary antibody solution, tissues were washed thrice with PBS for 10 minutes and reacted with the secondary antibody solution (biotinylated anti-IgG of Vectastain-Elite kit diluted 1:200, 0.3% Triton X-100) for an hour at the room temperature.

After reacting with the secondary antibody solution, tissues were washed thrice with PBS for 10 minutes and reacted with avidin-biotin peroxidase complex solution (A solution 1:100, B solution 1:100 of Vectastin-Elite kit, 0.3% Triton-100) for an hour at the room

temperature. 3,3'-diaminobenzidine tetrahydrochloride (Sigma) with 0.05M Tris buffer solution 0.02%, and H_2O_2 at 0.003% was used as a dye. Dye reaction was done at the normal temperature for 3-5 minutes and completed tissues were placed on the gelatin-coated slide and dried for 2 hours at the room temperature. Then it was made into transparency state with xylene and sealed with polymount.

For normal serum, goat serum was used for rabbit and horse serum was used for mouse.

Table I. The 1st antibody used for this Study

neuro-transmitter	manufacturer	dilution ratio
NPY	Incstar	1:2000
VIP	Transduction Lab.	1:2000

7. Tissue observation and image analysis

Staining intensity of the NADPH-d neuron, VIP neuron, and NPY neuron were measured[22] in gray scale using image analyzer (Multiscan, USA). Gray scale was set at 255 as white and 0 as black and dyed tissues were determined in this range at the fixed illumination. Each group was analyzed at least 10 regions with 200X optical microscope through CCD camera. Detailed structure and location were referred by the diagram charted by Paxinos and Watson[33](1997).

Ⅲ. Results

1. Effects of the Bee venom herbal acupucture on NADPH-diaphorase of the cerebral cortex

Staining intensity of NADPH -diaphorase neuron of the motor cortex in the saline solution group was measured at 130.0±2.6 versus 160.2±1.5 for the bee venom herbal acupuncture group, thus showed significant ($P<0.05$) increase. For the visual cortex, the saline solution group was at 112.4±5.5 compared to 159.7±2.0 of the bee venom group, therefore displayed significant increase. For the auditory cortex, the saline solution group figured at 127.5±5.2 versus 160.6±2.6 for the bee venom group, thus indicated significant increase. For the cingulate cortex, the saline solution group valued at 143.3±3.0 versus 156.0±3.0 for the bee venom group, once again demonstrated significant increase. For the retrosplenial

cortex, the saline solution group was measured at 147.0±2.2 compared to 159.3 ±2.1 for the bee venom group, therefore showed significant increase. For the perirhinal cortex, the saline solution group figured at 123.2±7.4 versus 163.3 ±3.7 for the bee venom group, once again displayed significant increase. For the somatosensory and insular cortex, the numbers were insignificantly higher in the bee venom groups. (Table II, Fig. 1)

2. Effects of the Bee venom herbal acupuncture on vasoactive intestinal peptide of the cerebral cortex

Staining intensity of vasoactive intestinal peptide neuron of the motor cortex in the saline solution group was measured at 135.4±6.3 versus 139.3±4.6 for the bee venom herbal acupuncture group, thus showed significant ($P<0.1$) increase. For the somatosensory cortex, the saline solution group was at 139.3± 4.6 compared to 129.8±2.9 of the bee venom group, therefore displayed significant ($P<0.1$) decrease. For the insular cortex, the saline solution group figured at 133.3±4.3 versus 145.9±2.3 for the bee venom group, thus indicated significant increase. For the retrosplenial cortex, the saline solution group was measured at 136.8±4.0 compared to 126.2 ±1.5 for the bee venom group, therefore showed significant decrease. For the perirhinal cortex, the saline solution group figured at 149.8±2.6 versus 138.4 ±3.8 for the bee venom group, and displayed significant decrease. (Table III, Fig. 2)

3. Effects of the Bee venom herbal acupuncture on Neuropeptide-Y of the cerebral cortex

Staining intensity of neuropeptide-Y neuron of the motor cortex in the saline solution group was measured at 119.5± 4.7 versus 136.5±4.7 for the bee venom herbal acupuncture group, thus showed significant ($P<0.1$) increase. For the visual cortex, the saline solution group was at 126.4±4.0 compared to 138.7±3.7 of the bee venom group, therefore displayed significant increase. For the cingulate cortex, the saline solution group valued at 110.2±3.9 versus 127.7±2.9 for the bee venom group, once again demonstrated significant increase. For the insular cortex, the saline solution group was measured at 129.1±4.4 compared to 116.3±5.5 for the bee venom group, therefore showed significant ($P<0.1$) decrease. For the retrosplenial cortex, the

Table II. Staining intensity of NADPH-diaphorase-positive neurons in the rat cerebral cortex

Area of cortex		AOD	
		Control	Sample
Neocortex	Motor cortex(MC)	130.0±2.6[a)]	160.2±1.5**
	Somatosensory cortex(SC)	138.1±2.5	142.3±2.2
	Visual cortex(VC)	112.4±5.5	159.7±2.0**
	Auditory cortex(AC)	127.5±5.2	160.6±2.6**
Allocortex	Cingulate cortex(CC)	143.3±3.0	156.0±3.0**
	Insular cortex(IC)	141.0±3.1	146.2±2.7
	Retrosplenial cortex(RC)	147.0±2.2	159.3±2.1**
	Perirhinal cortex(PC)	123.2±7.4	163.3±3.7**

a) : Mean±standard error

Statistical significance compared with the control data(*:P<0.1, **:P<0.05)

Control : Subcutaneous injection of normal saline at both LI 4.

Sample : Subcutaneous injection of Bee venom(1 : 100) at both LI 4.

AOD : average optical density

Fig. 1. Staining intensity of NADPH-diaphorase-positive neurons in the rat cerebral cortex

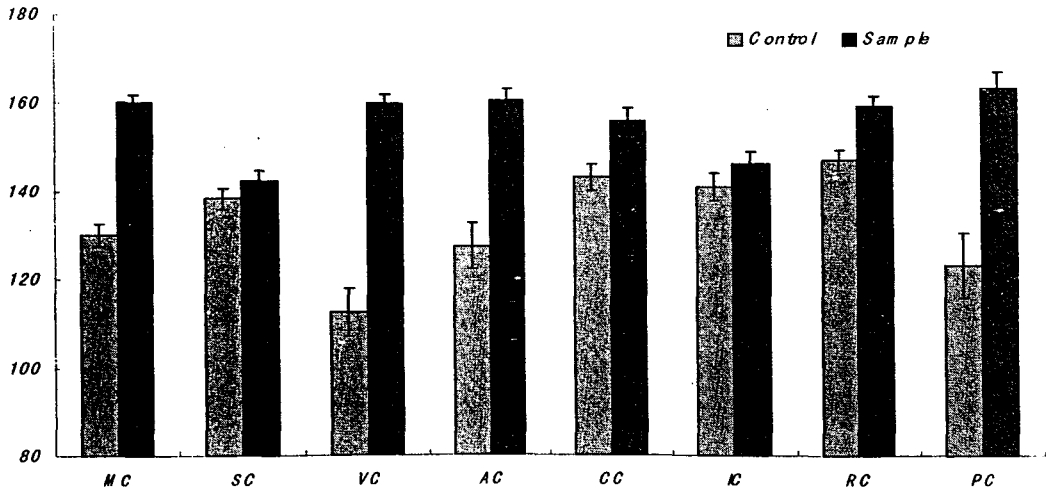


Table Ⅲ. Staining intensity of vasoactive intestinal polypeptide-positive neurons in the rat cerebral cortex

Area of cortex		AOD	
		Control	Sample
Neocortex	Motor cortex(MC)	135.4±6.3[a)]	137.2±3.0*
	Somatosensory cortex(SC)	139.3±4.6	129.8±2.9*
	Visual cortex(VC)	133.7±3.4	135.8±5.0
	Auditory cortex(AC)	147.0±3.7	145.4±2.4
Allocortex	Cingulate cortex(CC)	129.6±3.8	118.9±2.8
	Insular cortex(IC)	133.3±4.3	145.9±2.3**
	Retrosplenial cortex(RC)	136.8±4.0	126.2±1.5**
	Perirhinal cortex(PC)	149.8±2.6	138.4±3.8**

a) : Mean±standard error

Statistical significance compared with the control data(*:P<0.1, **:P<0.05)

Control : Subcutaneous injection of normal saline at both LI 4.

Sample : Subcutaneous injection of Bee venom(1 : 100) at both LI 4.

AOD : average optical density

Fig. 2. Staining intensity of vasoactive intestinal polypeptide-positive neurons in the rat cerebral cortex

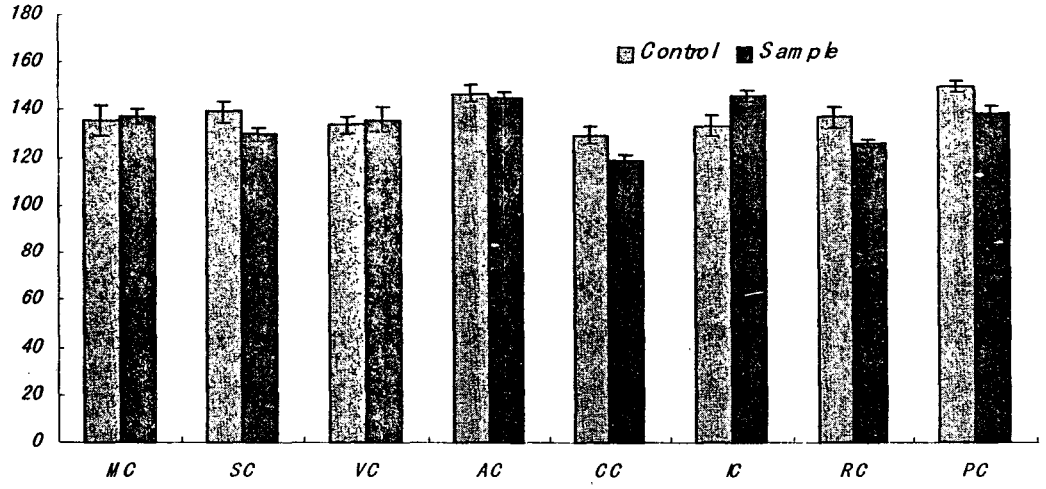


Table Ⅳ. Staining intensity of neuropeptide Y-positive neurons in the rat cerebral cortex

Area of cortex		AOD	
		Control	Sample
Neocortex	Motor cortex(MC)	119.5±4.7[a)]	136.5±4.7*
	Somatosensory cortex(SC)	139.9±2.9	130.4±5.2
	Visual cortex(VC)	126.4±4.0	138.7±3.7**
	Auditory cortex(AC)	141.9±3.0	135.3±4.6
Allocortex	Cingulate cortex(CC)	110.2±3.9	127.7±2.9**
	Insular cortex(IC)	129.1±4.4	116.9±5.5*
	Retrosplenial cortex(RC)	129.0±2.4	122.0±3.3*
	Perirhinal cortex(PC)	121.4±4.8	128.6±3.5

a) : Mean±standard error

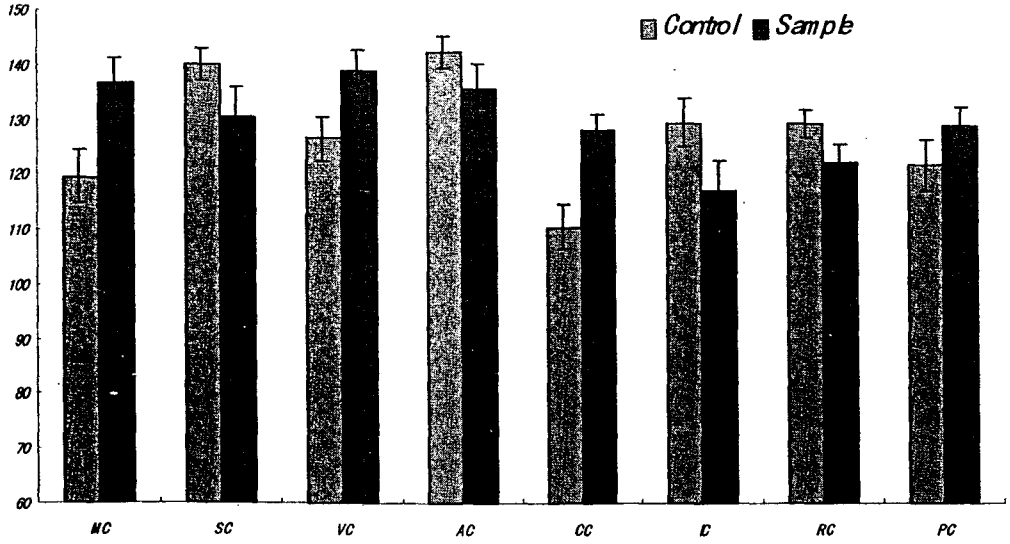
Statistical significance compared with the control data(*:P<0.1, **:P<0.05)

Control : Subcutaneous injection of normal saline at both LI 4.

Sample : Subcutaneous injection of Bee venom(1 : 100) at both LI 4.

AOD : average optical density

Fig. 3. Staining intensity of neuropeptide Y-positive neurons in the rat cerebral cortex



saline solution group figured at 129.0± 2.4 versus 122.0±3.3 for the bee venom group, and displayed significant ($P<0.1$) decrease. (Table Ⅳ, Fig. 3)

Ⅳ. Discussion

All the physiological activities of the human body are nonetheless governed by the central nervous system. And the neurons in the CNS communicate by exchanging information through synapse. Since the communication in the synapse area is carried out by the neurotransmitters and based on this fact, this experiment was conducted to measure the effects of the bee venom acupuncture on the CNS. The bee venom herbal acupuncture was given on the white rat on the points correspond to the human LI 4 and immunochemical effects were analyzed.

Traditional opinion on the neurotransmitters is that they are highly concentrated at the tip of nerve and relays the information by activating the receptors. Long distance communication requires activation by electricity but within closed circuit, the information is transferred without any electrical stimulation. Neurons of the brain relay information without any biological electricity and transfer the information from neuron A to neuron B via neurotransmitters[2-5), 19-21)].

This experiment is done to focus on three types of neurotransmitters that have been popularly studied in other researches.

Nitric Oxide (NO) is generated in the endothelial cells and by nitric oxide synthase (NOS) in L-arginine. NO plays as secondary neural messenger by acting in neuro-regulation and neuro-transmission.

But recent studies report that excess quantity of NO can be toxic. NO relieves tension of the vessels, lowers blood pressure, and suppress free radicals of the neurotransmitters in the CNS and PNS. To histologically examine the nerve cells with NOS, histochemistry of NADPH-diaphorase was used in the experiment. This is because of the principle that NOS reduces nitrobluetetrazolium (NBT) to water insoluble NBT formazan by activating NADPH- diaphorase[26)]. Results from this research yielded that the staining intensity of the motor cortex, visual cortex, auditory cortex, cingulate cortex, retrospenial cortex, and perirhinal cortex showed significant ($P<0.05$) increase in

the bee venom herbal acupuncture groups compared to the saline solution groups. Somatosensory cortex and insular cortex also demonstrated insignificant increase in the bee venom herbal acupuncture groups as well. Based on these facts, the bee venom herbal acupuncture plays a role in increasing NOS in the brain cortex.

VIP (vasoactive intestinal peptide) is a peptide that is similar to secretin, and unlike other gastric hormones, they are spread mainly in the neural cells instead of endocrine cells and plays as non-choline, non-adrenaline neurotransmitters.

Nerves containing VIP are easily found in the stomach, pancreas, gall bladder, and even in the brain. VIP is commonly known as the peptide with strong vasodilating activity, but also plays in inhibiting the release of gastric acid and promoting the release of HCO2- in the pancreas and bile from the gall bladder. Staining intensity of vasoactive intestinal peptide neuron of the motor cortex showed significant ($P<0.1$) increase in the bee venom herbal acupuncture group compared to the saline solution group.

For somatosensory cortex, the bee venom herbal acupuncture group displayed significant ($P<0.1$) decrease compared to the saline solution group. For insular cortex, the bee venom herbal acupuncture group demonstrated significant ($P<0.05$) increase in contrast to the saline solution group. Retrospenial and perirhinal contex showed significant ($P<0.05$) decrease in the bee venom herbal acupuncture groups compared to the saline solution groups. These results suggest that the bee venom herbal acupuncture mainly effects VIP in the allocortex region.

In the past, peptide line active susbstances were part of the gastric hormones or only existed in the endocrine glands, but current findings suggest that they are part of both gastric and central nervous system. Currently more than 100 active peptide factors are known to be part of the both systems. Among them, NPY (neuropeptide-Y) is found in high density in the CNS stored with catecholamine as well as spread in the stomach with pancreatic polypeptide (PP). It's known actions are vasoconstriction, increase the heart beat and myocardial constriction, and elevate blood pressure[2-5),19-21),32)].

From this experiment, staining intensity of neuropeptide-Y neurons of the motor cortex showed significant

($P<0.01$) increase in the bee venom herbal acupuncture group versus the saline solution group. For visual cortex and cingulate cortex, the bee venom herbal acupuncture groups displayed significant ($P<0.05$) increase compared to the saline solution groups. For insular cortex and retrospenial cortex, the bee venom herbal acupuncture groups displayed significant ($P<0.05$) decrease compared to the saline solution groups. From these data, the bee venom herbal acupuncture partially effects NPY, but couldn't link any consistency.

To conclude above experiment data, the bee venom herbal acupuncture significantly increase the NADPH -diaphorase in the brain cortex. For VIP and NYP, the bee venom herbal acupuncture either significantly increased or decreased depending on the cortex region, but couldn't associate any consistency.

This research had focused on the screening of neurotransmitters by the bee venom herbal acupuncture rather than specific study of technique. With the data from this experiment as a foundation, further researches should be done to collect more concrete information.

V. Conclusion

In order to study the effects of bee venom herbal acupuncture on the neurotransmitters of the rat brain cortex, herbal acupuncture with the bee venom group and normal saline group was performed bilaterally on the point corresponding to LI4 of the rat. The average optical density of the neurotransmitters from the cerebral cortex was analyzed 30 minutes after the herbal acupuncture with immunohistochemical method.

The results were as follows:

1. For NADPH-diaphorase, motor cortex, visual cortex, auditory cortex, cingulate cortex, retrosplenial cortex, and perirhinal cortex showed significant increase in the density in the bee venom groups compared to the saline solution groups.

2. For vasoactive intestinal peptide (VIP), significant changes were obtained in both the saline solution groups and the bee venom herbal acupuncture groups on the regions of insular cortex, retrosplenial cortex, and perirhinal cortex.

3. For neuropeptide-Y (NPY), the bee venom herbal acupuncture groups demonstrated significant increase in the regions of visual cortex and vingulate cortex, compared to the normal saline groups.

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