

The Effect of Acupuncture in Promoting Neurogenesis and Angiogenesis after Middle Cerebral Artery Occlusion in Rats

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[Abstract]

Objectives : This study was performed to choose more effective neuro-protective acupuncture point and to verify the effect of acupuncture in promoting neurogenesis and angiogenesis as a result of its neuro-vasculo-regenerative effect in middle cerebral artery occlusion model in rats.

Methods : By TTC staining we chose the most effective acupuncture point with neuro-protection. We randomly divided into four groups: Such as (1) sham group(with sham-operation), (2) sham+acupuncture group(with sham-operation), (3) middle cerebral artery occlusion group, (4) MCAO+AT group. Acupuncture procedure was performed for four days. Total RNA was extracted using TRIzol reagent, according to the manufacturer's instructions, and was purified using an RNaseasy mini kit. Immuno-histochemistry was performed using primary antibody mouse anti-BrdU, NeuN, Dcx, and VEGF.

Results : We found that ST₃₆ had the more neuroprotective effect than LI₁₁ and SP₃. The microarray analysis revealed that 54 genes were more expressed neurogenesis pathway in MCAO+AT group compared with MCAO group(fold changes greater than or equal to twofold change). 11 genes were more expressed angiogenesis pathway. And 7 genes were more expressed VEGF pathway.

Immuno-histochemistry revealed that cell proliferation, cell migration and cell maturation were increased.

Conclusions : This study demonstrated that acupuncture on ST₃₆ had neuro-protective and neuro-restorative effect in ischemic brain injuries. And its mechanism might be related to promote neurogenesis and angiogenesis. These results suggest that acupuncture have potential benefits for the treatment of ischemic stroke.

Key words :
 Acupuncture;
 Neurogenesis;
 Angiogenesis;
 Striatum;
 ST₃₆;
 MCAO

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I . Introduction

Experimental stroke studies demonstrate that focal cerebral ischemia promotes neurogenesis in the subventricular zone(SVZ) and induces SVZ neuroblast migration towards the ischemic boundary¹⁻³. And stroke induces angiogenesis and neurogenesis, which are coupled⁴⁻⁶. After stroke neuroblasts generated in the SVZ migrate to the ischemic boundary where angiogenesis occurs, and during migration neuroblasts are closely associated with cerebral vessels^{4,5}. Cell and pharmacologically based therapies have recently demonstrated that increased angiogenesis leads to enhancement of neurogenesis^{7,8}. These findings have led to a hope for a neurorestorative treatment of stroke, which aims to manipulate endogenous neurogenesis and angiogenesis thereby enhancing brain repair.

Acupuncture is utilized as a clinical treatment for various diseases in oriental medicine. It is known to possess many effects, such as analgesia, promotion of homeostasis, and changes in the microcirculatory network as well as improvements in brain circulation⁹⁻¹³. Acupuncture has been used to various kinds of movement dysfunction, including PD¹⁴.

Recent clinical studies have shown that electro-acupuncture(EA) can have functional benefits for motor function^{15,16}. In experimental studies, acupuncture has been demonstrated to have neuroprotective effects and improve cell proliferation^{17,18}, generation, migration and maturation of newly generated neuron in the striatum¹⁹. EA also can promote angiogenesis in brain of experimental cerebral ischemic rats through up-regulating the expression of angiogenesis factors and down-regulating the expression of anti-angiogenesis factors²⁰. And EA could activate the expression of several endogenous protective factors in ischemic brain, including bFGF²¹, GDNF²², and vascular endothelial growth factor (VEGF)²³, all of which have been demonstrated to facilitate neurogenesis in adult brain²⁴.

The striatum is a vulnerable region to the occlusion of middle cerebral artery, which is the most often

cause of stroke. Increase in the striatal neurogenesis and angiogenesis should be very important to promote capacity for neuronal replacement and self-repair in the adult brain after injury.

Therefore, we investigated whether acupuncture could promote neurogenesis and angiogenesis in the striatum of adult rat after a transient middle cerebral artery occlusion.

II . Materials and Methods

A. Animals and middle cerebral artery occlusion(MCAO)

Sprague-Dawley(SD) rats(Orient, Ltd, Seoul, Korea) were housed in an environmentally controlled room at 22 ± 2 °C, with a relative humidity of 55 ± 5 %, a 12 h light / dark cycle, and food and water ad libitum. The procedures involving experimental animals complied with the regulations for the care and use of laboratory animals of the animal ethics committee of Kyung Hee University.

Male rats weighing 300 ± 10 g were fasted overnight with free access to water. Rats were anesthetized with isoflurane (initiated with 5 % and maintained at 2 %) in 25 % O₂ / 75 % N₂. Rectal temperature was maintained at 37~38 °C throughout the surgical procedure by covering the animals with a heating pad. The right carotid bifurcation was exposed through a midline neck incision, and a filament, with a rounded tip and a distal cylinder of silicon rubber(0.30 mm in diameter), was introduced into the external carotid artery. A suture was then inserted at least 20 mm from the carotid bifurcation and was withdrawn 2 hours later to allow reperfusion.

B. Acupuncture procedure

In order to find the most effective acupuncture point with neuro-protection, we compared the effect

of several acupuncture points on the MCAO rat model. We used manual acupuncture at each acupuncture point. Stainless steel needles(0.20 mm diameter, 15 mm length, Dong-bang acu, Korea) was inserted. We divided the MCAO rats into four groups: Control(non-acupuncture), SP₃, LI₁₁, and ST₃₆(n=5 at each group). 30 minutes after MCAO, acupuncture treatment was performed at SP₃, LI₁₁, and ST₃₆. Rats were lightly immobilized, and acupuncture needles were inserted to a depth of 3mm at their respective acupuncture points bilaterally(Fig. 1)²⁵⁾, turned at a rate of two spins per second for 30s, and removed immediately afterward. This treatment continued at 24 hour intervals for 4 days. Rats were anesthetized 30 minutes after the last acupuncture treatment with diethyl ether and sacrificed. Brains were quickly removed and cut into six coronal sections, 2 mm thick, using a rat brain matrix(ASI® Instruments Inc, Warren, MI, USA). The sections were stained with 2 % TTC in saline at 37 °C for 30 min in the dark and photographed. Their images were analyzed using a computerized image analyzing system(Optimas 6.1, Media Cybernetics, Silver Springs, MD, USA).

The infarct area in each slice was calculated by subtracting the normal ipsilateral area from that of the contralateral hemisphere to reduce errors due to cerebral edema and was presented as a percentage relative to the area of the contralateral hemisphere. In parallel with infarct area, the edema volume in each slice was calculated by comparing the area of the ipsilateral hemisphere to the contralateral hemisphere, as described previously. In result, ST₃₆ showed the best neuroprotective effect for the MCAO rats(Fig. 2).

ST₃₆(located at the proximal one fifth point on the line from ST₃₅ to the anterior side of ankle crease)²⁵⁾, has been widely applied in oriental medicine for the treatment of the sequelae of stroke²⁶⁾. They were randomly divided into four groups: (1) sham group(sham-operation)(n=6), (2) sham+AT group (acupuncture on ST₃₆ after sham-operation)(n=6), (3) MCAO group(n=9), (4) MCAO+AT group (acupuncture on ST₃₆ after MCAO)(n=9). The above mentioned acupuncture procedure was performed for four days,



Fig. 1. The locations of acupuncture points

ST₃₆ is located at the proximal one fifth point on the line from ST₃₅ to the anterior side of ankle crease, LI₁₁ is located at the depression medial to the extensor carpi radialis, at the lateral end of cubital crease, SP₃ is located at the depression posterior to the first metatarso-phalangeal joint, on the medial side of hindfoot.

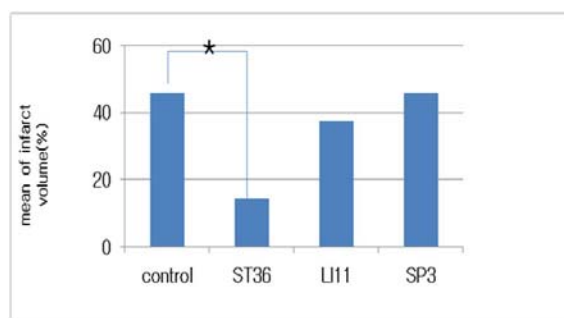


Fig. 2. TTC staining for measuring infarct volume(%) on four groups; control(non-acupuncture), ST₃₆, LI₁₁, and SP₃ acupuncture stimulation

Acupuncture on ST₃₆ significantly decreased infarct volume compared to the control group.

* : $p < 0.05$ compared to the control group.

C. Total RNA isolation and microarray analysis

Four days after the last acupuncture treatment, total RNA was extracted in the half brain by ischemic damage. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and was purified using an RNeasy mini kit(Qiagen, Valencia, CA, USA). Gene expression was analyzed using a Agilent's Gene Expression Hybridization Kit (genomictree, Seoul, Korea). Fluorescently labeled probes for the oligo microarray analysis were prepared using an Amino allyl Message Amp mRNA kit (Ambion Inc,

Austin, TX, USA). The microarray was designed with four replicates of each probe distributed across the array. Microarrays were assembled with hybridization caps and rehydrated with RNase-free water at 65 °C for 10 min. After rehydration, blocking solution was added, and the arrays were incubated at the hybridization temperature(45 °C) for 30 min. The cRNA mixtures were fragmented in fragmentation solution at 95 °C for 20 min. The fragmented cRNA sample was added to the hybridization solution and denatured (3 min at 95 °C). Blocking solution was removed from the hybridization chamber, and hybridization solution was applied to the arrays. Hybridizations were incubated for 18 hrs at 45°C. Two independent experiments were conducted.

The microarrays were imaged using a Agilent's DNA microarray scanner(Axon Instruments, Union City, CA, USA). Imaging was performed while the array was wet with 2X PBS under a LifterSlip glass coverslip(Erie Scientific, Portsmouth, NH, USA). Scanned images were analyzed using Feature Extraction software to obtain gene expression data. Gene expression was normalized by LOWESS regression.

D. Immunohistochemistry

To label proliferating cells, 5-bromo-2-deoxyuridine (BrdU)(50 mg/kg; Sigma-Aldrich, St Louis, MO) was injected intra-peritoneally twice daily for 4 consecutive days beginning 4 days after MCAO. Rats at various time points after ischemia-reperfusion (4 days) were perfused with saline followed by 4 paraformaldehyde in PBS. The brains were dissected out and postfixed overnight, cryoprotected in 20 sucrose and 30 sucrose, and embedded. Cryostat sections(40 μ m, cut coronally) were prepared. Animals were given i.p. injections of 5-bromo-2-deoxyuridine (BrdU)(50 mg/kg; Sigma-Aldrich, St Louis, MO) daily beginning until the day of sacrifice. After blocking in 1.0 % fish gelatin, the slides were incubated overnight at 4 °C with the primary antibody mouse anti-NeuN, DCX, VEGF (1 : 400~1 : 1,000). Slides

were washed and incubated with secondary antibody anti-rabbit IgG(1 : 200~1 : 1,000), washed in PBS, and mounted.

E. Statistics

Statistical analysis using SPSS 15.0 Data were expressed as the mean \pm SEM, and statistical significance was assessed by independent samples *t*-test. $P < 0.05$ was considered to be significant.

III. Results

A. Neuroprotective effect according to acupuncture points

Microscopic examination of TTC stained sections from rat brains showed the presence of infarction and neuronal death in striatum. Vascular regeneration existed around border of ischemic core in the striatum. Most neurons in striatum were shrunken and angular(Fig. 3). We observed that acupuncture on ST₃₆ and LI₁₁ groups decreased the infarction volume as compared with the control group. Acupuncture on ST₃₆ group was significantly different as compared with the control group($p < 0.05$)(Fig. 2). These results further support the hypothesis that acupuncture plays

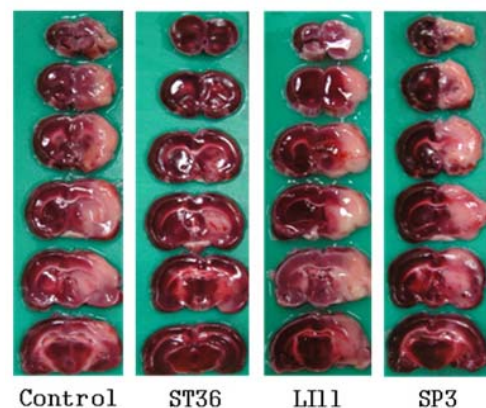


Fig. 3. TTC staining for infarct territory
The infarction site is changed with white color.

neuro-protective role in the pathophysiological process of the brain following ischemic injury.

B. Microarray

More than 44000 genes on the arrays were analyzed from MCAO rats. Among these genes, 54 genes were more expressed in neurogenesis pathway in MCAO+AT group compared with MCAO group (fold changes greater than or equal to twofold change) (Table 1). Some genes that have known to have direct function in neurogenesis were expressed. Such genes are glial cell line derived neurotrophic factor, insulin-like growth factor, doublecortin, fibroblast growth factor, epidermal growth factor²⁷⁻³¹. 11 genes were more expressed in angiogenesis pathway in MCAO+AT group compared with MCAO group (fold changes greater than or equal to twofold change) (Table 2). And 7 genes were more expressed in VEGF pathway in MCAO+AT group compared with MCAO group (fold changes greater than or equal to twofold change) (Table 3).

C. Cell proliferation in striatum of MCAO rats

In order to know whether acupuncture on ST₃₆ increased stroke-induced cell proliferation in ipsilateral striatum, we detected BrdU+ labeling cells. In sham group, BrdU+ cells were mostly located in the lateral ventricular zone. After transient MCAO, BrdU+ cells decreased in the ipsilateral striatum. Acupuncture on ST₃₆ could further enhance the BrdU+ cells in the striatum as compared with MCAO group (Fig. 4).

D. Cell migration in striatum of MCAO rats

Concerning the proliferating cells migrating into ipsilateral striatum, we used DCX+ labeling cells.

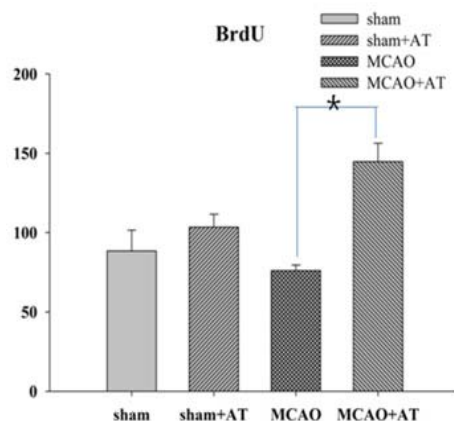
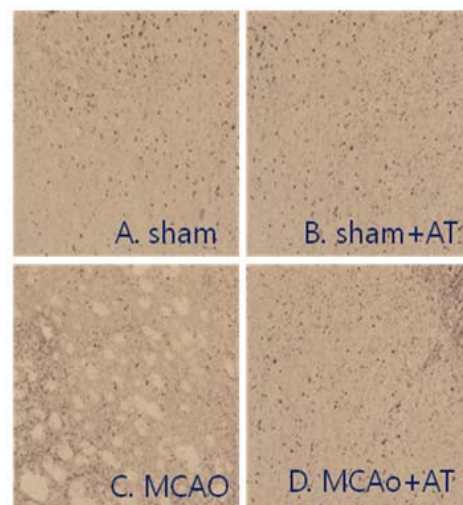


Fig. 4. Cell proliferation in the ipsilateral striatum of the MCAO rat

A : BrdU in sham group.
 B : BrdU in sham+AT group.
 C : BrdU in MCAO group.
 D : BrdU in MCAO+AT group.
 Number of NeuN positive cells/mm².
 MCAO group VS MCAO+AT group.
 * : $p < 0.05$, independent samples *t*-test.

Neuroblasts migrated from the SVZ to closely adjacent regions of the striatum. Migrating immature neurons (neuroblasts) were identified by the protein DCX, a specific marker for migratory or immature neurons in the adult brain. DCX+ cells were present in sham group in the striatum. And acupuncture increased DCX+ cells compared to sham group. But there was no significant difference. In MCAO group, DCX+ cells decreased as compared to sham group in 4 days after stroke. In focal stroke model, neuroblasts migrated from SVZ to infarction regions at days 7 and 14 days after stroke. Fig. 5 shows that acupuncture on

Table 1. List of Neurogenesis Expressed Genes(Upregulated Fold Changes at ≥ 2 about MCAO+AT Group / MCAO Group)

Genbank_ accession	Sham	Sham+AT	MCAO	MCAO+AT	ACCESSION gene name related genes species
NM_053398	1	0,088459395	0,17774552	0,85831726	Glial cell line derived neurotrophic factor family receptor alpha3
NM_182735	1	0,657018	0,27200347	1,0915835	Cyclin-dependent kinase inhibitor 1c(p57)
NM_178866	1	0,55649894	1,0126249	2,1160107	Insulin-like growth factor 1
XM_236203	1	2,5064547	1,2666227	2,5369658	Down syndrome cell adhesion molecule-like 1 (predicted)
NM_017129	1	1,4030789	1,2595829	2,6144564	Cardiotrophin 1
AF194995	0,99999	1,5293164	0,9579853	2,051878	Neuregulin 1
NM_053379	1	2,085573	0,54607177	2,7209532	Doublecortin
NM_024146	1	0,42243582	0,12037179	0,40319732	Fibroblast growth factor receptor 1
NM_019242	1	0,9230998	0,7963736	4,625277	Interferon-related developmental regulator 1
XM_219866	1	1,9206488	0,29265738	1,2238351	Laminin, alpha 2(predicted)
NM_019218	1	1,1079035	0,20404218	0,43733612	Neurogenic differentiation 1
NM_031507	1	2,0886865	0,38320062	2,2350621	Epidermal growth factor receptor
BG381007	1	0,43224496	0,26266298	0,5425529	Similar to m-ephrin-b3
BF389600	1	0,720613	0,37777412	0,97373426	Cdk5 activator-binding protein
XM_225316	1	0,7091513	0,41743532	1,756288	Doublecortin domain containing 2(predicted)
NM_080778	0,99999	0,79269516	0,4514761	0,9776451	Nuclear receptor subfamily 2, group f, member 2
NM_017310	1	1,6328987	0,223397	1,0008705	Sema domain, immunoglobulin domain(ig), short basic domain, secreted, (semaphorin) 3a
XM_343601	1	1,9093013	0,20531441	1,6816821	Paired box gene 3
NM_031130	1	0,16786191	0,14729787	0,39978763	Nuclear receptor subfamily 2, group f, member 1
NM_012609	1	6,6800075	1,877329	8,944215	Neurofibromatosis 1
XM_222370	1	3,3729553	0,33941767	2,9789555	Neurogenic differentiation 4(predicted)
XM_221311	1	0,14459403	0,02546906	0,06077912	Eph receptor b3(predicted)
CB544971	1	0,9348127	0,3053251	0,6125735	Sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (tm) and short cytoplasmic domain, (semaphorin) 5a(predicted)
AA964884	1	3,5135415	0,6166031	1,9189425	Peroxisome biogenesis factor 7
U73303	1	0,13331877	0,15334623	0,45722163	Myosin heavy chain 10, non-muscle
NM_173143	1	8,33583	0,34720516	1,6036181	Abl-interactor 2
AW920270	0,99999	0,5739745	0,25520453	0,8494899	Sema domain, immunoglobulin domain(ig), short basic domain
NM_012754	1	1,947251	2,0405753	4,6805935	Estrogen receptor 2 beta
XM_221672	1	1,4696556	0,70727956	2,0849888	T-cell lymphoma invasion and metastasis 1
AW914916	1	0,915328	0,24452055	0,5743325	Mitogen-activated protein kinase 8
NM_019621	1	1,1255364	0,21129292	0,96402615	Discs, lae homolog 4(drosophila)
NM_052807	1	1,7466171	1,0937675	13,632801	Insulin-like growth factor 1 receptor
NM_172009	1	0,50187445	0,1786244	0,49532697	Glycolipid-anchored form of acetylcholinesterase
XM_232343	1	0,8233645	0,14673495	0,41755953	Peroxisome biogenesis factor 5(predicted)

NM_053397	1	1,011879	0,46257895	1,1298032	Artemin
AA900438	1	1,1667264	0,21235807	0,78861064	Hairy and enhancer of split 5(drosophila)
AA998472	1	4,070161	0,51717705	3,8494198	Distal-less homeobox 5
XM_342392	1	3,269683	0,99288744	3,1496165	Notch gene homolog 1(drosophila)
AF250032	1	0,73503244	0,36600843	0,820463	Agrin
NM_022956	0,9999	0,64945334	0,6185042	2,381908	Barh-class homeodomain transcription factor
NM_001029911	1	0,45186132	0,035902496	0,12594922	Citron
NM_001005246	1	0,72486997	0,3376371	0,8119636	Dystrophin
NM_133652	1	1,3401704	1,2529843	4,300671	Chondroitin sulfate proteoglycan 5
NM_019326	0,9999	0,6748118	0,31811544	0,69890165	Neurogenic differentiation 2
NM_024147	1	0,73427546	0,86896205	2,101714	Ena-vasodilator stimulated phosphoprotein
NM_022282	0,9999	1,0002203	0,39535424	0,9592059	Discs, lae homolog 2(drosophila)
XM_225215	1	0,24104016	0,04140357	0,60248214	Sema domain, immunoglobulin domain(ig), trans-membrane domain(tm) and short cytoplasmic domain, (semaphorin) 4d(predicted)
XM_343260	0,9999	1,6281399	1,5748461	23,107918	Brain-specific angiogenesis inhibitor 1(predicted)
CB547594	0,9999	1,570386	1,1585444	16,939396	Amyotrophic lateral sclerosis 2(juvenile)
XM_342930	1	0,33687878	0,08833092	0,650927	Protein tyrosine phosphatase, receptor type, u
BF412087	1	1,3632429	0,72950554	2,421629	Neurexin 3
NM_022666	1	0,9095083	0,32877663	0,97600836	Glutamate receptor, metabotropic 4
NM_012754	1	1,947251	2,0405753	4,6805935	Estrogen receptor 2 beta
XM_221672	1	1,4696556	0,70727956	2,0849888	T-cell lymphoma invasion and metastasis 1

Table 2. List of Angiogenesis Expressed Genes(Upregulated Fold Changes at >2 about MCAO+AT Group / MCAO Group)

Genbank_ accession	Sham	Sham+AT	MCAO	MCAO+AT	Gene name
NM_017054	1	0,63193417	0,48948768	2,6571982	Thromboxane a2 receptor
XM_342392	1	3,269683	0,99288744	3,1496165	Notch gene homolog 1 (drosophila)
NM_024146	1	0,42243582	0,12037179	0,40319732	Fibroblast growth factor receptor 1
NM_017144	1	1,0961016	0,4615021	0,9534322	Troponin i, cardiac
CB544971	1	0,9348127	0,3053251	0,6125735	Sema domain, seven thrombospondin repeats(type 1 and type 1-like), transmembrane domain(tm) and short cytoplasmic domain, (semaphorin) 5a(predicted)
XM_343198	1	0,7007216	1,1999805	3,3781707	Elk3, member of ets oncogene family(predicted)
AF162914	1	2,7947986	0,71632135	2,3827035	Indian hedgehog homolog, (drosophila)
NM_022297	1	1,1850964	1,7409914	4,5279846	Dimethylarginine dimethylaminohydrolase 1
XM_225215	1	0,24104016	0,04140357	0,60248214	Sema domain, immunoglobulin domain(ig), trans-membrane domain(tm) and short cytoplasmic domain, (semaphorin) 4d(predicted)
NM_012609	1	6,6800075	1,877329	8,944215	Neurofibromatosis 1
BG670310	1	0,7987997	0,31926402	0,6747291	Transforming growth factor alpha

Table 3. List of VEGF Expressed Genes (Upregulated fold changes at >2 about MCAO+AT Group / MCAO Group)

Genbank_ accession	Sham	Sham+AT	MCAO	MCAO+AT	Gene name
BM389833	1	0.6009025	1.326665	5.0079107	Sphingosine kinase 2
NM_031585	1	0.8910768	0.360613	1.0704815	Phospholipase a2, group ib
NM_017176	1	1.2394303	0.6260453	4.147702	Phospholipase a2, group x
NM_022185	1	0.08356522	0.0578142	0.7502038	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2
XM_234904	1	2.4808056	1.0815164	52.322853	Src homology 2 domain-containing transforming protein c2(predicted)
NM_053481	0.99999	0.32224062	0.19358112	0.419349	Phosphatidylinositol 3-kinase, catalytic, beta polypeptide
NM_017093	1	0.14206854	0.305368	1.1306865	Thymoma viral proto-oncogene 2

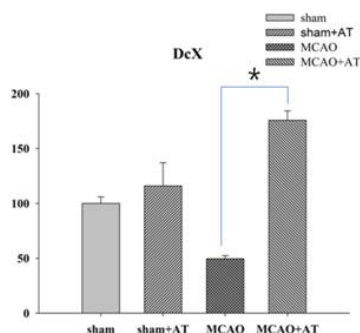
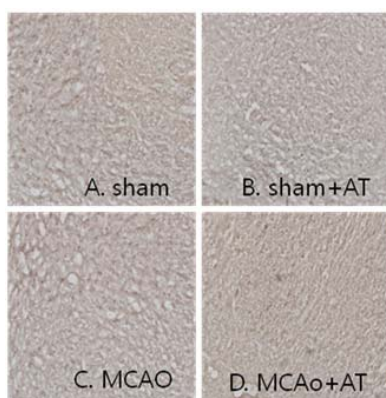


Fig. 5. Cell migration in the ipsilateral striatum of the MCAO rat

A : DCX in sham group,
 B : DCX in sham+AT group.
 C : DCX in MCAO group,
 D : DCX in MCAO+AT group.
 Number of NeuN positive cells/mm².
 MCAO group VS MCAO+AT group.
 * : $p < 0.05$, independent samples *t*-test.

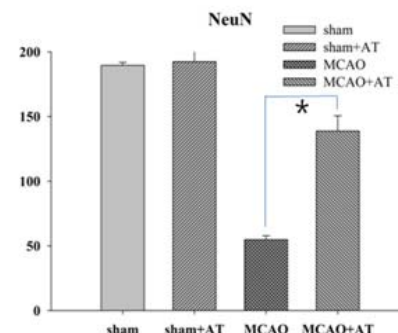
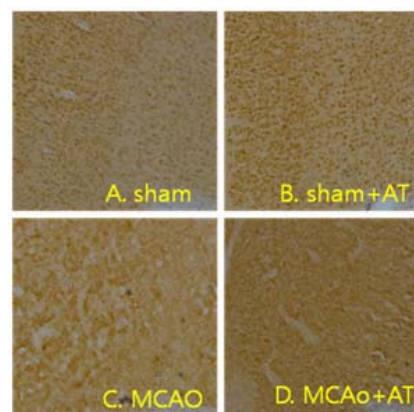


Fig. 6. Cell maturation in the ipsilateral striatum of the MCAO rat

A : NeuN in sham group,
 B : NeuN in sham+AT group.
 C : NeuN in MCAO group,
 D : NeuN in MCAO+AT group.
 Number of NeuN positive cells/mm².
 MCAO group VS MCAO+AT group.
 * : $p < 0.05$, independent samples *t*-test.

ST₃₆ could significantly enhance the DCX+ cells in the striatum as compared with MCAO group.

E. Cell maturation in striatum of MCAO rats

In order to verify if the migration cells can be matured into new neurons, we used NeuN+ labeling cells. The initial migration of immature neurons into areas of damage after stroke is present in the striatum. Despite this initial migration in the first week after stroke, a relatively small number of newly-born neurons matured and survived in the long-term. Generation of newly-born neurons after stroke was associated with functional recovery. These surviving neurons displayed immunohistochemical staining for mature neuronal proteins. Mature neurons were identified by the NeuN, a specific marker for mature neurons in the adult brain. In sham group, NeuN+ cells

were present in the striatum. In MCAO group, NeuN+ cells decreased as compared to sham group in 4 days after stroke. In focal stroke model, neurons were damaged by ischemic shock. Fig. 6 shows that acupuncture on ST₃₆ could further enhance the NeuN+ cells in the striatum as compared with MCAO group.

F. Angiogenesis in striatum of MCAO rats

VEGF(vascular endothelial growth factor) is the most important mitogen in the process of angiogenesis³²⁾. Stroke leads to angiogenesis in the ischemic hemisphere through the vascular endothelial growth factor system. To determine whether acupuncture promotes angiogenesis, we counted VEGF+ cells. Compared with sham group, MCAO group increased the VEGF+ cells 4 days after MCAO. And acupuncture on ST₃₆ increased VEGF+cells in striatum as compared with MCAO group(Fig. 7). But there was no significant difference.

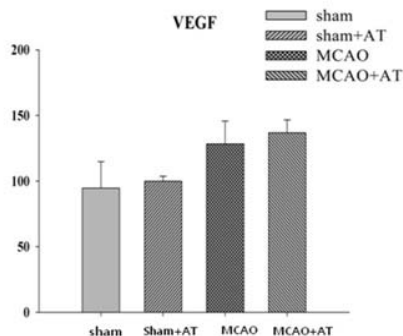
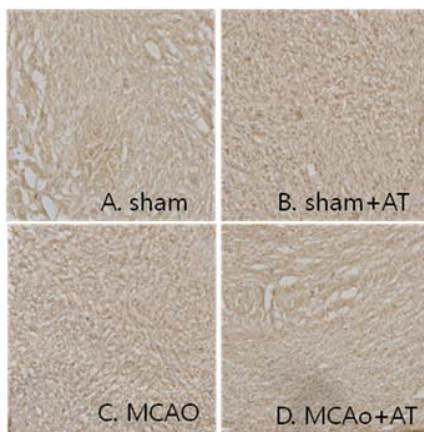


Fig. 7. Angiogenesis in the ipsilateral striatum of the MCAO rat

A : VEGF in sham group.

B : VEGF in sham+AT group.

C : VEGF in MCAO group.

D : VEGF in MCAO+AT group.

number of VEGF positive cells/mm².

MCAO group VS MCAO+AT group.

* : $p < 0.05$, independent samples *t*-test.

IV. Discussion

The purpose of this study was to investigate whether acupuncture has the neuro-restorative effect on the striatum in the early stages after cerebral ischemia. To find the most effective acupuncture point, we compared several acupuncture points on the stroke model. In the traditional acupuncture books, such as *Dongeuibogam* and *Chingudaesung*, acupuncture points on GB, LI, and ST meridians were often used to treat stroke³³⁾. In those acupuncture points, we chose the most commonly used acupuncture points LI₁₁ and ST₃₆ on stroke model in the recent study^{17,18,34-36)} and chose SP₃ which is not commonly used in treatment of stroke. This acupuncture screening test, ST₃₆ showed the best neuroprotective effect for the stroke model. In oriental medicine, ST₃₆ has been widely applied for the treatment of the sequelae of stroke²⁶⁾.

Acupuncture on ST₃₆ alleviates ischemia-induced apoptosis and presents possible therapeutic potentials in the recovery from ischemic cerebral injuries¹⁷. Acupuncture treatment on ST₃₆ suppressed the hemorrhage-induced increase in lesion size and apoptotic neuronal cell death in the striatum³⁶. This results suggests that Acupuncture treatment on ST₃₆ may affect cell proliferation after ischemic injury¹⁸.

Microarray techniques allow the detection and quantification of differential expression for thousands of genes simultaneously in a single experiment³⁷. We examined by using microarray analysis to search the neuro-restorative effect of acupuncture on ST₃₆. Other research showed that 24 hr after reperfusion, most genes were up-regulated in the ischemic penumbra³⁸. We found corresponding genes especially for neurogenesis, angiogenesis and VEGF. We found that MCAO+AT group is more expressed genes than MCAO group (greater than two-fold change). Our data shows that fifty four genes were expressed in pathway of neurogenesis. In previous reports, many molecular contenders stimulated neurogenesis in ischemic-brain, including basic fibroblast growth factor (bFGF)²⁷, epidermal growth factor (EGF)²⁸, glial cell line-derived neurotrophic factor (GDNF)³¹. Our data shows that these factors' gene is expressed. In addition, eleven genes were expressed in pathway of angiogenesis. And seven genes were expressed in pathway of VEGF. These genes in relation to neurogenesis and angiogenesis have been altered their expression levels by acupuncture on ST₃₆. We understand that the pathway of acupuncture effect is related to neurogenesis and angiogenesis in the ischemic rats.

Stroke triggers dynamic changes of the G1 phase of actively dividing SVZ cell cycle resulting in early expansion of a neural progenitor pool and later neuronal differentiation, which leads to augmentation for neurogenesis³⁹. It is known that BrdU is an analog of thymidine and can be incorporated into DNA of cells during the S phase and thus has been used to investigate cell proliferation⁴⁰. The number of BrdU-positive cells peaks from 7 to 14 days after ischemia and returns to control level 3~5 weeks after

ischemia⁴¹. In our present study, acupuncture on ST₃₆ significantly increased the number of BrdU-labeled cells in the striatum of rats compared with MCAO group. It suggests that acupuncture on ST₃₆ may promote cell proliferation.

Migrating immature neurons were identified by DCX, a specific marker for migratory or immature neurons in the adult brain⁴². Neuroblasts migrate from the SVZ to closely adjacent regions of the striatum. Within degenerating striatum, DCX+ cells appear to migrate along both astrocytes and blood vessels^{1,2}. Neuroblasts transiently expressed during about 2~3 weeks in neuronal progenitors⁴². This study, we found that acupuncture significantly increased the number of DCX+ neurons in striatum of rats 4 days after MCAO, compared with MCAO group. The results indicate that acupuncture has the potential to promote neuroblast migration to striatum after cerebral ischemia.

Substantial initial migration after stroke, a relatively small number of newly born neurons mature and survive in the long-term. These surviving neurons display immunohistochemical staining for mature neuronal proteins including the neuronal nuclear antigen NeuN^{43,44}. The generation of newly-born neurons after stroke is associated with functional recovery⁵. We found that acupuncture significantly increased the number of NeuN+ neurons in striatum of rats 4 days after MCAO, compared with MCAO group. The results indicate that acupuncture has the potential to promote newly born maturation after cerebral ischemia.

Neurogenesis and angiogenesis are closely linked in the germinal zones of the adult brain⁵. Neural progenitor cells lie in close proximity to endothelial cells, bursts of angiogenesis occur at the same time as neurogenesis, and endothelial cells secrete soluble factors that regulate neuronal differentiation *in vitro*⁴⁵. These findings suggest that the micro-environment surrounding blood vessels, which is termed vascular niche, may play an important role in neurogenesis in the adult brain⁵. In order to investigate the mechanism by which acupuncture promotes angiogenesis, the expression of VEGF

(vascular endothelial growth factor) was detected in the present study. VEGF initially discovered as an angiogenic and vascular permeability factor⁴⁶⁾, is now recognized in playing a new role as a neurotrophic factor⁴⁷⁾. It has been shown that endogenous neuronal VEGF is upregulated in rat brains after transient ischemia, which plays a compensatory neuro-protective role⁴⁸⁾. In recent studies, electroacupuncture significantly increased VEGF mRNA expression in 2 hours and reached to the peak 24 hours after ischemic brain²⁰⁾. Our data showed that acupuncture increased the VEGF 4 days after MCAO+AT group as compared with MCAO group. Thus we suppose that acupuncture-induced increase of VEGF may also promote angiogenesis in the brain after MCAO.

V. Conclusion

This study demonstrated that acupuncture on ST₃₆ has neuro-protective and neuro-restorative effects in ischemic brain injuries. And its mechanism might be related to promoting neurogenesis and angiogenesis. These results suggest that acupuncture has potential benefits for the treatment of ischemic stroke.

VI. References

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