

Original Articles

Acupuncture Suppresses Intrastratial Hemorrhage-Induced Neuronal Cell Death and Proliferation in Rats

Nam-Hun Cho, Dong-suk Park, Jae-Dong Lee

Department of Acupuncture and Moxibustion, College of Oriental Medicine, Kyung Hee University

Objectives : Intracerebral hemorrhage (ICH) is one of the most devastating types of stroke. The effect of acupuncture on the intrastratial hemorrhage-induced neuronal cell death and cell proliferation in rats is examined.

Methods : Cell death and cell proliferation in rats was investigated via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and immunohistochemistry for caspase-3 and 5-bromo-2'-deoxyuridine (BrdU).

Results : Results showed that apoptotic cell death in the striatum and cell proliferation in the hippocampal dentate gyrus significantly increased following intrastratial hemorrhage in rats, and that acupunctural treatment at the Zusanli acupoint suppressed the hemorrhage-induced increase in apoptosis in the striatum and cell proliferation in the dentate gyrus.

Conclusions : It is suggested that acupunctural treatment, especially at the Zusanli acupoint, may aid recovery following central nervous system sequelae following ICH. (*Korean J of Oriental Med* 2003;24(4):127-135)

Key Words: acupuncture, intrastratial hemorrhage, apoptosis, cell proliferation, Zusanli acupoint

Introduction

Intracerebral hemorrhage (ICH) is one of the most devastating types of stroke, which commonly occurs in the striatum, thalamus, cerebellum, and pons²⁰. ICH is associated with severe neurological deficits and a considerable mortality rate. In hemorrhagic stroke, brain damage occurs through multiple mechanisms. These mechanisms include direct tissue destruction, space-occupying effect of the hematoma, ischemic damage to adjacent tissue, clot-derived toxic factors, and edema formation³⁴.

Acupuncture is utilized as a clinical treatment for various diseases in Oriental medicine and possesses many positive effects such as the promotion of homeostasis, improvement in brain circulation, pain control, and neuromodulatory function in the central nervous system^{2,14,31,35}. In recent studies, acupunctural treatment has been shown to be particularly effective for improvement in the symptoms of cerebrovascular accident^{8,9,35}.

Brain cell death after ICH may be mediated in part by apoptotic mechanisms²⁷. Apoptosis, also known as programmed cell death, is a form of cell death that serves to eliminate dying cells in proliferating or differentiating cell populations¹⁷, thus playing a crucial role in normal development and tissue homeostasis³⁰. On the other hand, inappropriate or excessive apoptosis

Received 10 November 2003; revised 17 November 2003; accepted 21 November 2003

Correspondence to: Nam-hun Cho, Kyung Hee Oriental Medicine Hospital, Seoul, 130-701, Korea; Tel: 82-2-958-9198, E-mail: cnhdoc@freecchal.com

has been implicated in several types of neurodegenerative disorders, including ischemia^{16,32}.

Apoptosis is known as an important form of ICH-induced cell death^{10,27}, and activation of caspases-3 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive cells have also been reported following ICH¹⁰.

TUNEL staining is a widely used method in the detection of DNA fragmentation, a characteristic of apoptotic cell death⁹. Caspases, which make up a family of cysteinyl proteases encompassing 14 members, are essential players in apoptotic cell death both as initiators (caspase-2, -8, -9, and -10) and executioners (caspase-3, -6, and -7)²⁹. It has also been reported that neuronal cell death in the parenchyma occurs by an apoptotic mechanism in ICH rats and is associated with the induction of caspase-3 in cells adjacent to the hematoma¹⁰.

Neurogenesis encompasses cell proliferation, survival, migration, and neuronal differentiation. The birth of new cells in the hippocampus, which plays an important role in learning and memory, continues in adult mammals including humans^{5,7}. Cell proliferation in the hippocampal dentate gyrus is known to be enhanced by learning, serotonin, estrogen, antidepressants, N-methyl-D-aspartate (NMDA) receptor antagonists, and physical exercise. Adrenal steroids, stress, and aging are known to inhibit it. Generation of new cells in adults is also enhanced by pathological events such as seizures and ischemic insult^{24,28}. Such regulation of cell proliferation occurring during pathological situations is thought to be a compensatory response to lesion-induced cell death in the brain²⁴.

In this study, the effect of acupuncture on the intrastriatal hemorrhage-induced neuronal cell death and cell proliferation in rats was investigated via TUNEL assay and immunohistochemistry for caspase-3 and 5-bromo-2'-deoxyuridine (BrdU).

Materials and Methods

1. Experimental animals and treatment

Experimental procedures were performed in accordance with the animal care guidelines of the National Institute of Health (NIH) and the Korean Academy of Medical Sciences. Male Sprague-Dawley rats weighing 200 ± 10 g (6 weeks in age) were used in this experiment. The rats were housed under controlled temperature (20 ± 2 °C) and lighting (07:00 to 19:00 h) conditions with food and water made available ad libitum before and after surgery. Animals were randomly divided into four groups ($n = 5$ in each group): the sham-operation group, the hemorrhage-induction group, the Zusanli-acupunctured hemorrhage-induction group, and the non-acupoint-acupunctured hemorrhage-induction group.

In the acupunctured groups, acupunctural treatment was given to each animal twice daily (10:00 a.m. and 5:00 p.m.) for 7 days, starting on the third day of the experiment. Stainless acupuncture needles of 0.3 mm diameter were bilaterally inserted about 2-4 mm depth into the locus of the Zusanli acupoint (ST 36), located 5 mm lateral and distal to the anterior tubercle of the tibia for the Zusanli acupoint, or into the hip for the non-acupoint, and left in place for 20 min.

2. Induction of hemorrhage

For induction of hemorrhage, rats were anesthetized with pentobarbital sodium (40 mg/kg, ip; Sigma Chemical Co., St. Louis, MO) and placed in a stereotaxic frame.

Through a hole drilled in the skull, a 26-gauge needle was implanted into the striatum at the following coordinates: 2.6 mm lateral to midline, 0.7 mm anterior to coronal suture, depth 4.5 mm deep from the surface of the brain, and 1 μ L of saline containing 0.2 U collagenase (Type 4; Sigma Chemical Co.) was infused

over 1 min. The needle remained in place for an additional 3 min following the infusion, and then was slowly withdrawn. Animals of the sham-operation group received an equivalent dose of physiological saline with the same method.

3. Tissue preparation

The rats were sacrificed on the 9th day of the experiment, immediately after finishing acupuncture treatment. Animals were weighted and overdosed with Zoletil 50[®] (10 mg/kg, ip; Vibac Laboratories, Carros, France). After a complete lack of response was observed, the rats were transcardially perfused with 50 mM phosphate-buffered saline (PBS), and fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB, pH 7.4). The brains were dissected and postfixed in the same fixative overnight and transferred into a 30% sucrose solution for cryoprotection. Serial coronal sections of 40 μ m thickness were made using a freezing microtome (Leica, Nussloch, Germany).

4. Nissl staining

For the determination of lesion size, Nissl staining was performed as previously described²⁵. Digitalized images of the Nissl-stained cells were obtained from the field of view under a light microscope (Olympus, Tokyo, Japan). The lesion area of the collagenase injection side was determined using an Image-Pro[®] Plus image analyzer (Media Cybernetics Inc., Silver Spring, MD). The lesion volume in the striatum (%) was calculated as follows: hemorrhage-induced lesion size (collagenase injected side) / intact striatum size (contralateral side) x 100.

5. TUNEL staining

TUNEL assay was performed using In Situ Cell Death Detection Kit[®](Roche, Mannheim, Germany) as

per the manufacturer's protocol¹³. Sections were post-fixed in ethanol-acetic acid (2:1) and rinsed. Then the sections were incubated with proteinase K (100 μ g/ml), rinsed, incubated in 3% H₂O₂, permeabilized with 0.5% Triton X-100, rinsed again, and incubated in the TUNEL reaction mixture. The sections were rinsed and visualized using Converter-POD with diaminobenzidine (DAB). Mayer's hematoxylin (DAKO, Glostrup, Denmark) was used for counter-staining. The slides were air-dried overnight at room temperature, and cover slips were mounted using Permount[®]. The numbers of TUNEL-positive cells were assessed quantitatively in the four fields (400 \times 400 μ m in each field) within the striatum adjacent to the hematoma previously described²⁷.

6. Caspase-3 immunohistochemistry

For visualization of caspase-3 expression, caspase-3 immunohistochemistry was performed¹³. Sections were drawn from each brain and incubated overnight with mouse anti-caspase 3 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and then for another 1 h with biotinylated mouse secondary antibody. Bound secondary antibody was then amplified with Vector Elite ABC kit[®] (Vector Laboratories, Burlingame, CA). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.02% 3,3-diaminobenzidine (DAB) and the sections were finally mounted onto gelatin-coated slides. The numbers of caspase-3-positive cells were assessed quantitatively in the four fields (250 \times 250 μ m in each field) within the striatum adjacent to the hematoma as previously described³⁷.

7. BrdU immunohistochemistry

For detection of newly generated cells in the dentate gyrus, BrdU-specific immunohistochemistry was performed as previously described¹⁹. Sections were first permeabilized by incubation in 0.5% Triton X-100 in

PBS for 20 min, then pretreated in 50% formamide-2 x standard saline citrate (SSC) at 65 °C for 2 h, denatured in 2 N HCl at 37 °C for 30 min, and rinsed twice in 100 mM sodium borate (pH 8.5). Afterwards, the sections were incubated overnight at 4 °C with BrdU-specific mouse monoclonal antibody (1:600; Roche, Mannheim, Germany). The sections were then washed three times with PBS and incubated for 1 h with a biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA). Then the sections were incubated for another 1 h with avidin-peroxidase complex (1:100; Vector Laboratories, Burlingame, CA, USA). For visualization, the sections were incubated in 50 mM Tris-HCl (pH 7.6) containing 0.02% DAB, 40 mg/ml nickel chloride, and 0.03% hydrogen peroxide for 5 min, and the sections were finally mounted onto gelatin-coated slides. The slides were air dried overnight at room temperature, and coverslips were mounted using Permount®.

8. Data analysis

Statistical significance of difference was determined by one-way analysis of variance (ANOVA) followed by Duncan's post-hoc analysis, and results were expressed as mean ± standard error mean (S.E.M.). Difference was considered significant at $p < 0.05$.

Results

1. Effect of acupuncture on the lesion size in the striatum

Photomicrographs of the lesion area in the striatum are presented in Fig. 1. The neuronal lesion size was $3.50 \pm 0.50\%$ compared to intact striatum in the sham-operation group, $46.64 \pm 5.10\%$ in the hemorrhage-induction group, $32.50 \pm 7.10\%$ in the Zusanli-acupunctured hemorrhage-induction group, and $48.50 \pm 5.70\%$ in the non-acupoint-acupunctured hem-

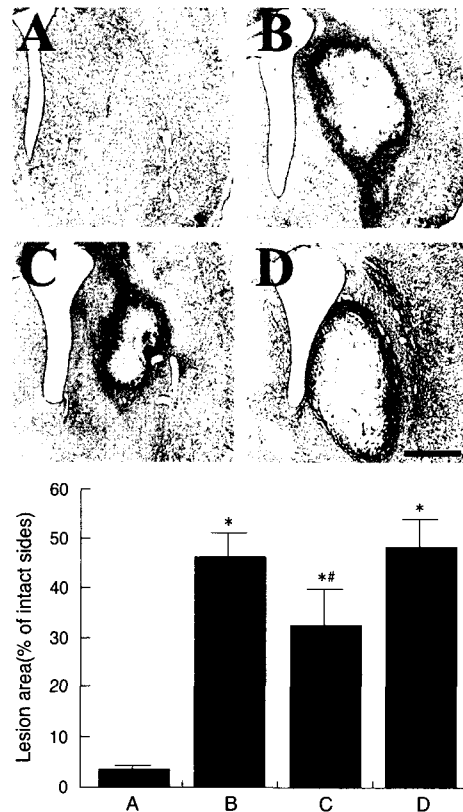


Fig. 1. Effect of acupuncture on the size of intrastriatal hemorrhage-induced lesions.

Upper: Photomicrographs showing Nissl staining in the striatum. Scale bar represents 800 μm .

Lower: Mean size of lesion area compared to normal striatum in each group.

Values are represented as a mean \pm S.E.M.

* represents $p < 0.05$ compared with the sham-operation group.

** represents $p < 0.05$ compared to the hemorrhage-induction group.

A. the sham-operation group; B. the hemorrhage-induction group, C. the Zusanli-acupunctured hemorrhage-induction group, D. the non-acupoint-acupunctured hemorrhage-induction group.

orrhage-induction group. Present results show that intrastriatal injection of collagenase increased lesion size in the striatum and that acupunctural treatment at the Zusanli acupoint significantly suppressed the intrastriatal hemorrhage-induced neuronal cell death.

Effect of acupuncture on the number of TUNEL-positive cells in the striatum Photomicrographs of the TUNEL-positive cells in the striatum are presented in

Fig. 2. The number of TUNEL-positive cells was $43.75 \pm 6.25/\text{mm}^2$ in the sham-operation group, $1030.46 \pm 71.52/\text{mm}^2$ in the hemorrhage-induction group, $625.00 \pm 36.13/\text{mm}^2$ in the Zusanli-acupunctured hemorrhage-induction group, and $1028.57 \pm 33.37/\text{mm}^2$ in the non-acupoint-acupunctured hemorrhage-induction group.

Results show that intrastratial hemorrhage enhanced the number of TUNEL-positive cells in the striatum and that acupunctural treatment at the Zusanli acupoint significantly suppressed the intrastratial hemorrhage-induced increase in apoptotic cell death.

2. Effect of acupuncture on the number of caspase-3-positive cells in the striatum

Photomicrographs of the caspase-3-positive cells in the striatum are presented in Fig. 3. The number of caspase-3-positive cells was nearly zero in the sham-operation group, $1957 \pm 66.60/\text{mm}^2$ in the hemorrhage-induction group, $1325.97 \pm 43.33/\text{mm}^2$ in the Zusanli-acupunctured hemorrhage-induction group, and $2121.15 \pm 64.43/\text{mm}^2$ in the non-acupoint-acupunctured hemorrhage-induction group. Results show that intrastratial hemorrhage enhanced caspase-3 expression in the striatum and that acupunctural treatment at the Zusanli acupoint significantly suppressed the intrastratial hemorrhage-induced increase in apoptosis.

3. Effect of acupuncture on the number of BrdU-positive cells in the dentate gyrus

Photomicrographs of the BrdU-positive cells in the striatum are presented in Fig. 4. The mean number of BrdU-positive cells in the dentate gyrus was $122.07 \pm 4.54/\text{mm}^2$ in the sham-operation group, $161.00 \pm 5.77/\text{mm}^2$ in the hemorrhage-induction group, $132.89 \pm 5.05/\text{mm}^2$ in the Zusanli-acupunctured hemorrhage-induction group, and $156.96 \pm 10.60/\text{mm}^2$ in the non-acupoint-acupunctured hemorrhage-induction group.

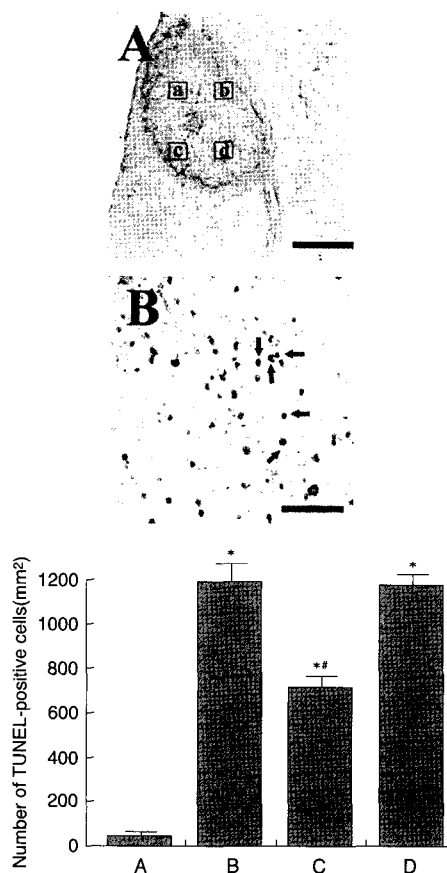


Fig. 2. Effect of acupuncture on DNA fragmentation in the striatum.

Upper: Photomicrographs showing immunostaining for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The number of TUNEL-positive cells in the fields a, b, c, and d were counted. Arrows indicate TUNEL-positive cells. Scale bar represents $800 \mu\text{m}$ (A) and $25 \mu\text{m}$ (B).

Lower: Mean number of TUNEL-positive cells in each group. Values are represented as mean \pm S.E.M.

* represents $p < 0.05$ compared with the sham-operation group.

represents $p < 0.05$ compared to the hemorrhage-induction group.

A. the sham-operation group, B. the hemorrhage-induction group, C. the Zusanli-acupunctured hemorrhage-induction group, D. the non-acupoint-acupunctured hemorrhage-induction group.

Results show that cell proliferation in the dentate gyrus was enhanced in the hemorrhage-induction group and that acupunctural treatment at the Zusanli-acupoint suppressed the intrastratial hemorrhage-induced increase in cell proliferation.

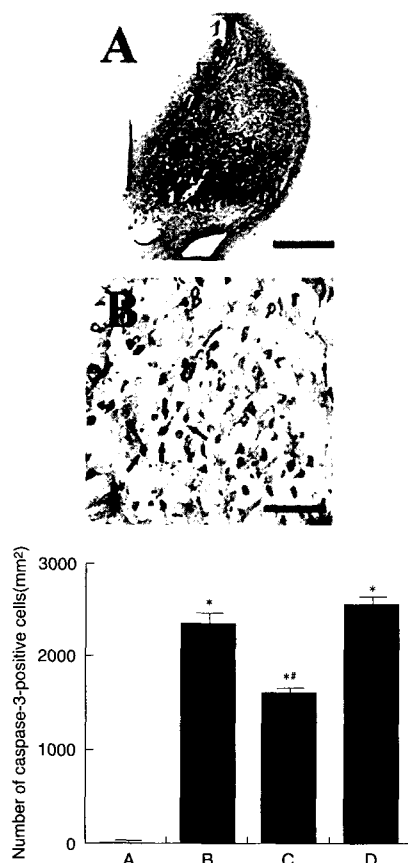


Fig. 3. Effect of acupuncture on caspase-3 expression in the striatum.

Upper: Photomicrographs showing immunostaining for caspase-3. The numbers of caspase-3 positive cells in the field a, b, c, and d were counted. Arrows indicate caspase-3 positive cells. Scale bar represents 800 μm (A) and 25 μm (B). Lower: Mean number of caspase-3-positive cells in each group. Values are represented as a mean \pm S.E.M. * represents $p < 0.05$ compared with the sham-operation group. # represents $p < 0.05$ compared to the hemorrhage-induction group. A. the sham-operation group; B. the hemorrhage-induction group, C. the Zusanli-acupunctured hemorrhage-induction group, D. the non-acupoint-acupunctured hemorrhage-induction group.

Discussion

In this study, the numbers of TUNEL-positive and caspase-3-positive cells in the striatum increased significantly following ICH, indicating that intrastriatal hemorrhage leads to apoptotic neuronal cell death in the

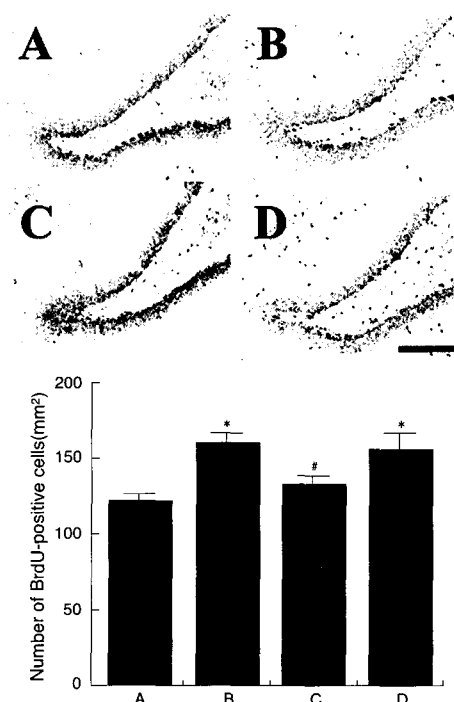


Fig. 4. Effect of acupuncture on cell proliferation in the dentate gyrus.

Upper: Photomicrographs showing immunostaining for 5-bromo-2'-deoxyuridine (BrdU)-positive cells. Sections were stained for BrdU (black) and neuronal nuclei (NeuN; brown). Scale bar represents 100 μm . Lower: Mean number of BrdU-positive cells in each group. Values are represented as a mean \pm S.E.M. * represents $p < 0.05$ compared with the sham-operation group. # represents $p < 0.05$ compared to the hemorrhage-induction group. A. the sham-operation group; B. the hemorrhage-induction group, C. the Zusanli-acupunctured hemorrhage-induction group, D. the non-acupoint-acupunctured hemorrhage-induction group.

striatum. Apoptosis appears to play a key role in neuronal cell death following ischemic stroke^{22,23}. Caspase-3 plays a pivotal role in apoptotic cell death³ and is activated in the ischemia-reperfusion injury¹. In addition, apoptotic cell death can be assessed by TUNEL staining, which detects DNA fragmentation^{29,30}. Del Bigio⁴ reported that intrastriatal hemorrhage in rats induces neuronal cell death, and it was also reported that apoptosis is closely implicated in ICH-induced neuronal cell death^{10,27}.

In this study, increased cell proliferation was observed in the hippocampal dentate gyrus following intraatrial hemorrhage. Ischemia-induced increment in cell proliferation is known as a compensatory response to excessive apoptotic cell death^{24,33}.

Acupuncture is known to possess a neuroprotective effect against cerebral ischemia in monkeys⁸ and gerbils³⁵. One of the most impressive effects of acupuncture is a rapid recovery from the complications of stroke¹². Acupuncture increases cerebral blood flow and improves microcirculation¹⁵ and has been used as a safe and effective therapeutic modality in treating acute cerebral hemorrhage, particularly effective in recovering limb paralysis and speech disturbance induced by ICH²¹.

This study showed that acupunctural treatment at the Zusanli acupoint suppresses increase in the TUNEL-positive cell number and caspase-3 expression in the striatum following intraatrial hemorrhage. Based on these results, it is quite possible for the acupunctural effect on reducing lesion size to be ascribed to inhibition of apoptotic cell death. Acupunctural treatment also suppressed the hemorrhage-induced increase in cell proliferation in the dentate gyrus, which can be ascribed to the reduction of apoptotic cell death by acupuncture. These effects of the acupunctural treatment were not observed at the non-acupoint. Present results reveal that acupuncture at the Zusanli acupoint alleviates intraatrial hemorrhage-induced apoptosis, suggesting that acupunctural treatment may aid in the recovery following central nervous system sequelae following ICH.

Conclusion

Intracerebral hemorrhage (ICH) is one of the most devastating types of stroke. The effect of acupuncture on the intraatrial hemorrhage-induced neuronal cell

death and cell proliferation in rats was investigated via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and immunohistochemistry for caspase-3 and 5-bromo-2'-deoxyuridine (BrdU). The results showed that apoptotic cell death in the striatum and cell proliferation in the hippocampal dentate gyrus significantly increased following intraatrial hemorrhage in rats and that acupunctural treatment at the Zusanli acupoint suppressed the hemorrhage-induced increase in apoptosis in the striatum and cell proliferation in the dentate gyrus. It is suggested that acupunctural treatment, especially at the Zusanli acupoint, may aid in the recovery following central nervous system sequelae following ICH.

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