THE EFFECTS OF VARIOUS FORMS OF ACUPOINT STIMULATION ON CEREBRAL ISCHEMIC RAT MODEL

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

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CERTIFICATE OF ORIGINALITY

I hereby declare that the thesis is my own work and that, to the best of my knowledge and belief, it produces no material previously published or written, nor material which has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

YIP KA KEUNG

Abstract of the thesis entitled

"The effect of various forms of acupoint stimulation on cerebral ischemic rat model"

Submitted by

YIP KA KEUNG

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Cerebrovascular stroke is the second leading cause of death in the world which account for 9.9% of the total number of death. There are two major types of cerebral stroke: ischemic and hemorrhagic. Ischemic stroke is the occlusion of cerebral arteries where hemorrhagic stroke is the accumulation of blood within the brain region. Nearly 80 % of the cerebral stroke cases are ischemia stroke. Acupuncture is traditional Chinese medicine (TCM) with the history over 2500 years. It treats patients by insertion and manipulation of needles in the specific points, acupoints, of the human body. Acupoints are the location to reflect the pathological changes and receive the stimulation for treatment of disease. Previous studies revealed that the use of acupuncture in cerebral ischemia provides favorable result. As acupuncture follows the theory of meridian, the current study hypothesizes that the critical factor of treatment is the acupoint stimulation whatever the forms of stimulation is. Therefore, the study aims at investigating the effectiveness of different forms of acupoint stimulation on providing neuroprotection under cerebral ischemic condition using rat model. Since the selection of acupoint is also the determining factor of treatment, different acupoint will be employed in the study to investigate the acupoint-specific effect. Three forms of acupoint stimulation were employed: electroacupuncture (EA), high voltage electrical stimulation (HVES) and laser irradiation. Multiple pre-ischemia EA stimulation at non-acupoint (NA), GB 20 and ST 36

were applied to examine the effect on cerebral ischemia by measuring two anti-apoptotic molecules: Bcl-2 and transforming growth factor beta-1 (TGFβ-1). Both Bcl-2 and TGFβ-1 were found to be significantly increased in the ST 36 groups. However in the GB 20 groups, significant increase was only observed in TGFβ-1. No significant elevation of the level of TGFβ-1 was observed in the NA groups, but a significant enhancement was indicated in the production of Bcl-2. The data suggest that multiple pre-ischemia EA at ST 36 was effective in conferring neuroprotective effect on the brain by means of upregulation of Bcl-2 and TGFβ-1. Stimulation group of GB 20 and NA provided less favorable result indicating acupoint specificity in the treatment of cerebral ischemia. In the second study, the effect of multiple pre-ischemia acupoint HVES on minimizing the cerebral neuronal damage was investigated by stimulating acupoints ST 36, LI 10 or ST 36 with LI 10. The production of inducible nitric oxide synthase (iNOS) and malondialdehyde (MDA) were attenuated at ST 36 group but not in LI 10 group. Combinational use of acupoint of ST 36 and LI 10 did not provide positive effect. The third study is laser irradiation. The effects of single low-energy laser irradiation (LLI) on the levels and activity of various anti- and pro-apoptotic factors following ischemia were investigated. After the induction of transient cerebral ischemia by unilateral occlusion of the middle cerebral artery for 1 hour followed by reperfusion, LLI was then directed on the ashi point for varying lengths of duration (1, 5 or 10 min at an energy density of 2.64 J/cm2, 13.2 J/cm2 and 24.6 J/cm2, respectively). Ashi point is traditional acupoint which is reported pain by patient when pressing. The expression levels of Akt, pAkt, BAD, pBAD, Bcl-2, caspase 9 and caspase 3 activity were measured 4 days post-injury. LLI was demonstrated to protect the brain by up-regulating Akt, pAkt, pBAD and Bcl-2 expression and down-regulating caspase 9 and caspase 3 expression following transient cerebral ischemia. In conclusion, the current study demonstrated that acupoint stimulation in various types may be effective in cerebral ischemia neuroprotection.

However, the protective effect may be acupoint specific. Selection of acupoint is important in the efficacy of treatment. Clarification of individual and combined application of acupuncture point is needed in further research.

PUBLICATIONS ARISING FROM THE THESIS

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2. Yip KK, Lo SCL, So KF, Leung MCP, Poon DMY. Pre-ischemia electro-acupuncture potentiates the expression of Bcl-2 and transforming growth factor beta-1 in rat brains. Am J Chin Med. [Submitted]
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LIST OF ABBREVIATIONS

ABBREVIATION	FULL NAME
ADL	Activity of Daily Living
Akt, pAkt	Serine/threoninekinase, phosphorylated form of Akt
BAD, pBAD	Bcl-2 associated death protein, phosphorylated form of BAD
Bax	Bcl-associated X protein
Bcl-2	B-cell lymphoma/leukemia-2
Beta-actin	β-actin
BID	BH3 interacting-domain death agonist
Caspace	Aspartate-specific cysteine protease
DEVD	Asp-Glu-Val-Asp
EA	electroacupuncture
HVES	high voltage electrical stimulation
ICE	IL-1 beta converting enzyme
iNOS	inducible nitric oxide synthase
LLI	Low-energy laser irradiation
MAS	Motor Assessment Scale
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MDA	malondialdehye
m-RNA	messenger ribonucleic acid
mRS	modified Ranki Scale
NA	non-acupoint
NHP	Nottingham Health Profile
NIHSS	National Institutes of Health Stroke Scale
NOS	nitric oxide synthase
$paCO_2$	arterial partial carbon dioxide
paO_2	arterial partial oxygen
pNA	p-nitroaniline
rtPA	Recombinant tissue plasminogen activator
SDS-PAGE	SDS-polyacryamide gel electrophoresis
TBS-T	Tris-Buffered Saline and Tween 20
TCM	Traditional Chinese medicine
TENS	transcutaneous electrical stimulation
TGFβ-1	Transforming growth factor beta 1
tPA	Tissue plasminogen activator

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Chapter 1

Introduction

1.1 Cerebral stroke

According to the statistics obtained by World Health Organization (2003), cerebrovascular disease (stroke) is the second leading cause of death in the world. Stroke mainly happens in adults and aged people. In 2005, it was estimated that about 5.7 million people died because of stroke in the world which equivalent to 9.9% of the total number of death. Two-thirds of the incident was come from developing countries and nearly 40% of cases were under the age of 70. Although Hong Kong is regarded as a developed country, the incident of cerebrovascular disease is also serious. The disease ranked the fourth of the leading causes of death in 2009 (Department of Health, The Government of Hong Kong Special Administrative Region, 2011). The death rate was 49.2 per 100,000 population in the territory. Even the patient is survived from cerebral stroke, the public health system has to suffered from the huge economic burden to afford the continuous support to the surviving disabilities. Therefore, numerous research efforts have been made in developing effective treatment for cerebral stroke to answer the social demand of the issue.

1.2 Classification of stroke

According to the World Health Organization, cerebral stroke is defined as “rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause of other than of vascular origin” (WHO MONICA Project Investigators, 1988). There are two major types of cerebral stroke: ischemic and hemorrhagic.

Ischemic stroke is the occlusion of cerebral arteries. The occlusion which happens directly at the site of blocking is thrombotic ischemic stroke while the occlusion of cerebral vessels due to the distal forming clot is embolic ischemic stroke. Besides the aforementioned types of ischemic stroke which the superficial cerebral vessels usually involved, there are lacunar ischemic stroke which the deep penetrating cerebral vessels is blocked. All these kinds of ischemic stroke account for nearly 80% of the cerebral stroke cases.

Hemorrhagic stroke is the accumulation of blood within the brain region. Intracerebral hemorrhage is mainly due to arteriolar hypertensive disease while subarachnoid hemorrhage is mainly due to the breaking of aneurysms at the bifurcations of large cerebral arteries.

1.3 Risk factor of cerebral stroke

There are two types of risk factors for cerebral stroke: controllable and uncontrollable. Controllable factors include life style and medical risk factors. Life style risk factors are tobacco use and smoking, alcohol use, physical inactivity and obesity which can easily be changed while medical risk factors like high blood pressure, atrial fibrillation, high cholesterol, diabetes, atherosclerosis and circulation problems need medical professionals help. Uncontrollable factors include age, sex, race and family history. Being over age 55, being male, being African American, Hispanic or Asian/Pacific Islander, or having a family history of stroke or transient ischemic attack have higher risk in getting cerebral stroke.

1.4 Treatment of cerebral stroke

As ischemic stroke is caused by blood clotting of the intracranial artery, the key therapeutic strategy focuses on removing the thrombus by pharmacologically (thrombolysis) or mechanically (thrombectomy) in order to restore blood flow resulted in minimizing the number of death of cerebral cells. Anti-platelet drugs such as aspirin, clopidogrel and dipyridamole are usually prescribed to prevent clot enlargement or new clot formation.

Thrombolysis is employed the drug tissue plasminogen activator (tPA) to dissolve the clot and unblock the cerebral artery. tPA is a serine protease that catalyzes the activation of the zymogen plasminogen by converting it to plasmin. To date, only intravenous administration of recombinant tPA (rtPA) has been proven to be effective. It is the first and only approved agent by the Food and Drug Administration in 1996 based on the finding of the National Institute of Neurological Disorders and Stroke rt-PA Stroke Study (The National Institute of Neurological Disorders, and Stroke rt-PA Stroke Study Group, 1995). 624 patients with ischemic stroke were recruited in the study and were treated with intravenous injection of placebo or rtPA (0.9 mg/kg, maximum 90 mg) within 3 hours of symptom onset. The outcome measures of the study were: 1) the neurological improvement at 24 hours as indicated by complete neurological recovery or an improvement of 4 points or more on the NIH Stroke Scale and 2) complete or nearly complete neurological recovery at 3 months after stroke. Favorable results were achieved in 31% to 50% of patients treated with rtPA as compared with 20% to 38% of patients given placebo. However, there was no improvement of the mortality rate in the two treatment groups.

Thrombectomy is directly removal of clot by surgical method. A catheter is inserted into the femoral artery and directed into the intracranial circulation to deliver a corkscrew-like device which is used to trap the clot. The treatment is only performed in patient who is not able to take pharmacological therapy or the therapy is ineffective. Recently, angioplasty and stenting was applied in the surgery of clot removal (Mori et al., 2000).

Treatment of hemorrhagic stroke requires neurosurgical evaluation to find out the location and the cause of bleeding. Accurate diagnosis is important as the anticoagulant and antithrombotic drugs used in treating ischemic stroke worsen the degree of intracerebral hemorrhage.

1.5 Acupuncture

Acupuncture is traditional Chinese medicine (TCM) with the history over 2500 years. Acupuncture treats patients by insertion and manipulation of needles in the specific points, acupoints, of the human body (Leake and Broderick, 1998; Tai, 2002).

The central concept of acupuncture bases on the theory of TCM that disease is the result of the imbalance of Yin and Yang (known as negative and positive force) and Wu Xing (known as the five phases or elements, earth, water, fire, wood and metal) (Lu et al., 2002). The imbalance is due to the disturbance of the flowing of qi, the vital energy, through the channel called meridians. Under the theory of TCM, there are totally eleven organs in the body under two main catergories: Yin and Yang. Yin organs include lung, heart, spleen, kidney, and liver. Yang organs include large intestine, small intestine, stomach, urinary bladder, gallbladder and sanjiao. Those organs are linked by 12 meridians and two

vessels.

Acupoints are the location to reflect the pathological changes and receive the stimulation for treatment of disease. They are located along the skin with the properties of more peripheral nerves, lower electrical impedance and higher electrical potential than other parts of the skin (Rosenblatt, 1981; Leake and Broderick, 1998). Three types of acupoints are classified: Channel points, Extra points and Ashi points (Xiao and Mu, 2000). There are totally 361 channel points which are located through the meridian channels. Extra points are the points not belong to the meridian channels and a total of 50 Extra points are commonly employed in clinical practice. Ashi points are the points that the patients report pain when pressing.

1.6 Clinical studies of the effectiveness of acupuncture treatment on cerebral stroke

Johanasson et al. (1993) investigated the effectiveness of acupuncture in 78 patients averaged 76 years old with severe hemiparesis. Patients in control group received daily physiotherapy and occupational therapy while patients in treatment group received additional acupuncture therapy twice a week for 10 weeks. Patients given additional acupuncture treatment obtained significant improvement in balance, mobility, activities of daily living, quality of life, and days spent at hospitals/nursing homes than those in control group.

Hu et al. (1993) studied the effectiveness of acupuncture treatment on cerebral ischemic patients with routine supportive treatment and additional acupuncture therapy. A total of 30 patients, aged 46 to 74, with the onset of symptoms within 36 hours were employed in the

study after appropriate screening. Patients in treatment group were given acupuncture 3 times weekly for 4 weeks. A significantly improvement of neurological outcome was observed in the treatment group on day 28 and day 90 and the improvement was greatest in patients with a poor neurological score at the onset. This study illustrated the therapeutic effect of acute cerebral stroke on younger subjects.

Apart from the acute phase effectiveness, subacute stage application also provided favorable result. Kjendahl et al. (1997) recruited 45 cerebral ischemic patients with median post-stroke time of 40 days. With additional classical acupuncture treatment 3 to 4 times weekly for 6 weeks in the acupuncture group, the Motor Assessment Scale (MAS) for stroke patients, Sunnaas Index of Activity of Daily Living (ADL) and Nottingham Health Profile (NHP) were significantly better in the assessment at 6 weeks and approximately 1 year after discharge of the hospital.

There were, of course, some negative findings of acupuncture treatment of cerebral stroke. With the successful of previous finding of acupuncture therapy on cerebral stroke (Johanasson et al., 1993), the author performed a comparative study of acupuncture, electroacupuncture (EA) and transcutaneous electrical stimulation (TENS) on 150 patients with moderate or severe impartment after stroke (Johanasson et al., 2001). Treatment was launched between 5 and 10 days after the onset. A total of 20 treatments were given twice a week for 10 weeks. All the intervention groups demonstrated an improvement in assessment (ADL proficiency, mobility, walking speed, and quality of life), but no significant differences between the groups were observed. It should be noticed that the control group in the study received stimulation which was used as the same in the TENS group only the intensity (0.4 mA) was set below the perception threshold (no skin sensation and no visible

muscle contractions). The existence of other kinds of receptor may account for the improvement of the control group (Kawakita, 1993).

Sze et al. (2002) performed acupuncture on patients started stroke within 15 days. After passing the assessment of haemodynamic stability, dementia and admission Barthel Index (within 3 to 15), patients were assigned either control group or acupuncture group. Control group received standard treatment while the acupuncture group was given additional acupuncture therapy on 10 main acupoints: Jianyu-LI15, Quchi-LI11, Shousanli-LI10, Hegu-LI4, Waiguan-TE5, Huantiao-GB30, Yanglingquan-GB34, Zusanli-S36, Jiexi-S41, and Kunlun-B60 based on the TCM theory. No significant difference was obtained in Fugl-Meyer assessment, Barthel Index, and Functional Independence Measure, respectively, at weeks 0, 5, and 10 of two groups. Although the authors claimed that the selection of those acupoints under the theory of meridian can facilitate the blood flow, result obtained from limited scientific study of the direct relation between acupoint and physiology is questionable.

1.7 Experimental studies of the effectiveness of acupuncture treatment on cerebral stroke

Zhai et al. (1993) used middle cerebral artery occlusion (MCAO) model in rat to investigate the effect of acupuncture. They found that ischemic volume was only occupied 6.7% of the whole brain in the acupuncture group compared to 20.7% of the whole brain was infracted in the control group at 36 hours post-injury.

Kang et al. (2010) investigated the neuroprotective effect of acupuncture in the MCAO-

induced ischemia rat model. Rats were divided into 2 groups: MCAO and MCAO plus acupuncture. Acupuncture stimulation to both GB 34 and GB 39 was given immediately after reperfusion. The infarction volume was significantly decreased in the treatment group (16.4 ± 4.8%) compared with the injury group (39.9 ± 10.2%). The number of neuron-specific nuclear protein-positive cells in the treatment group was significantly increased in striatum (from 42.3 ± 12.6% to 67.0 ± 3.8%) and in the motor cortex (from 45.8 ± 5.8% to 67.0 ± 3.8%).

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Chapter 2

Aim of the study

The use of acupuncture in cerebral ischemia provides favorable result. In traditional Chinese medicine, acupuncture follows the theory of meridian. The current study hypothesizes that the critical factor of treatment is the acupoint stimulation whatever the forms of stimulation is. Therefore, the study aims at investigating the effectiveness of different forms of acupoint stimulation on providing neuroprotection under cerebral ischemic condition. Since the selection of acupoint is also the determining factor of treatment, different acupoint will be employed in the study to investigate the acupoint-specific effect.

Chapter 3

Materials and Methods

3.1 Grouping of animal

All experimental procedures were approved by the Hong Kong Polytechnic University animal ethical committee. Sprague-Dawley rats were employed in the study. Firstly, rats were used to measure common physiological parameters in blood obtained from the femoral artery 15 minutes before and during cerebral ischemia. Then the subjects were assigned to the corresponding group for measurement of the different parameters. In general they were evenly divided into four experimental groups: an uninjured group (rats with general anesthesia without stimulation), an injured group (rats with cerebral ischemia but without treatment), a non-acupoint (NA) group (rats with stimulation at non-acupoints) and a treatment group (rats with acupoint stimulation). Acupoint stimulation was carried out on alternate days before the induction of cerebral ischemia in pre-ischemia application studies. In post-ischemia study, acupoint stimulation was carried out just after the surgically induced cerebral ischemia. Different number of stimulation was employed for investigating the duration effect of the studied stimulation.

3.2 Induction of cerebral ischemia and reperfusion

Transient focal cerebral ischemia was induced by occluding the right middle cerebral artery (MCA) for 1 hour followed by reperfusion as previously described by Chen et al. (1986), with modifications (Leung et al., 2002). Briefly, male Sprague–Dawley rats were anaesthetized with an intraperitoneal injection of ketamine (70 mg/kg, Alfasan Nederland B.V.) and xylazine (7 mg/kg, Alfasan Nederland B.V.). A scalp incision was made at the

midpoint between the right eye and ear, and the right temporalis muscle was dissected and retracted to expose the zygoma and squamosal bones. A 5 mm x 5 mm window was prepared with a constantly cooled surgical drill (Mototool, Dremel). The right MCA was occluded for 1 hour by a surgical clip and then released to allow reperfusion. The removed bone was replaced and the temporalis muscle and overlying skin were sutured. Throughout the surgical procedure, the rectal temperature was monitored and maintained at approximately 37 °C with the use of an overhead lamp. Fifteen minutes before and after occlusion of MCA, femoral arterial blood was sampled for pH, arterial partial oxygen (paO_2) and carbon dioxide ($paCO_2$) pressure measurements using the Blood Gas and Electrolyte System (ABL 505, Radiometer, USA). Following the procedure, the rats recovered at room temperature (21-23 ºC) with *ad libitum* food and water.

3.3 Pre-ischemia electro-acupuncture (EA)

A 30-minute pre-ischemia EA treatment was applied bilaterally at corresponding acupoints by an acupunctoscope device (Model G6805-2, Smeif, Shanghai, China) (Voltage: 0.7 V, frequency: 2 Hz, duration: 0.5 ms) as previously described (Siu et al., 2004). The stimulation was carried out on alternate days before the induction of cerebral ischemia.

3.4 Pre-ischemia acupoint high voltage electrical stimulation (HVES)

Pulsed pre-ischemia acupoint HVES (0.4 ms) was delivered bilaterally at corresponding acupoint through a pair of fixed round-shaped electrodes measuring 2mm in diameter for 30 minutes (Model ZDL-4, Nanjing, China) at voltage 1,000 V with frequency 2.5 Hz. Intensity was set at the level of muscle contraction just initiated. Ultrasound gel was

applied for better conductivity (Chattanooga Group, Modialloc Victoria Australia). Treatment was taken corresponding to the treatment group with frequency of 3 times per week before the induction of cerebral ischemia.

3.5 Laser irradiation

Immediately following right middle cerebral artery occlusion (MCAO), a 660-nm laser beam (average power 8.8 mW with a 1-min energy density of 2.64 J/cm^2 and pulse frequency of 10 kHz) (Omega Excel Laser, Omega Laser Systems LTD, U.K.) was directed through the burr hole onto the cerebrum from a distance of 5 mm. The laser beam was on a gallium aluminum arsenide probe with a divergence adaptor and delivered a spot area of 20 mm^2. The application site was similar to the traditional acupoint, ah-shi point, where the patient feel painful when acupuncture needle inserts. The laser light exposure was 1, 5 or 10 min, delivering 2.64 J/cm^2 (n=8), 13.20 J/cm^2 (n=8) or 26.40 J/cm^2 (n=6), respectively.

3.6 Sample collection

Rats were sacrificed 4 days after ischemia-reperfusion with an overdose ketamine and xylazine mixture. The sampling point was determined by considering the maximum nitric oxide synthase (NOS) activity after the induction of cerebral ischemia (Leung et al. 2002). Transcardial perfusion was performed with 0.38 % sodium citrate in normal saline for 10 minutes followed by 0.9 % sodium chloride for 5 minutes. The harvested right hemisphere of the brain was weighted and homogenized in 20 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 3000 g for 10 minutes at 4 ^{o}C and the supernatant was collected for analysis.

3.7 Lipid peroxidation assay

Lipid peroxidation and oxidative stress at cellular level was determined by titrating the brain tissue of the end-product, malondialdehye (MDA), using an immunoassay kit (Oxis International, Inc., Portland, OR, USA), which reflected the formation of a chromophore with MDA molecule (Erdelmeier et al., 1998). Each sample mixture was composed of 50 µl of supernatant, 50 µl of 20 mM Tris-HCl (pH 7.4) and 325 µl of 10.3 mM N-methyl-2-phenylindole in acetonitrile. To assay for the quantity of MDA, sample mixture was added with 75 µl of 15.4 M methanesulfonic acid and incubated at 45 ^{o}C for 40 minutes. After incubation, samples were cooled on ice and their absorbance was read at 586nm. The unit was defined as µM per mg homogenate protein.

3.8 Quantification of TGFβ-1, Bcl-2, iNOS, Akt, pAkt, BAD, pBAD, Bcl-2 and Caspase 9 Proteins by Western Blot Analysis

The protein concentration of homogenate prepared from the right cerebral hemisphere was measured with the Bio-Rad Protein Assay (Bio-Rad Laboratories). In brief, 100 µg of soluble protein was resolved by SDS-polyacryamide gel electrophoresis (SDS-PAGE) and the separating gels were electro-blotted overnight at 4 ºC. The blots were incubated overnight at 4 ºC with rabbit antibodies against rat TGFβ-1, Bcl-2, iNOS, Akt, pAkt, Bad, pBad, Bcl-2 and caspase 9 (1:200 dilution, 0.5 µg/ml, Santa Cruz Biotechnology, Inc.). After reacting with Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.), chemiluminescence was captured by the UVP-Chemi System (UVP, Inc.), and signal intensities (expressed as ng/mg total protein) were analyzed using Lab-Works software (UVP, Inc.). Select blots were directly reprobed with anti–β-actin antibody (1:5000; Sigma Chemical Co.) and the resulting signal intensity was used to confirm equal sample loading

and even transfer. The expression of apoptotic parameters is defined as ng/mg protein.

3.9 Functional assay of caspase 3 activity

Caspase 3 specifically cleaves at the C-terminal side of the aspartate residue in the target sequence DEVD (Asp-Glu-Val-Asp). The CaspACE Assay System Colorimetric Kit (Promega) was used to measure caspase 3 activity. Each 40-μl sample was incubated with 32 μl caspase assay buffer, 2 μl dimethyl sulfoxide, 10 μl 100 mM dithiothreitol and 2 μl colorimetric substrate (DEVD-pNA) for 4 hours at 37 °C. This substrate was labeled with chromophore p-nitroaniline (pNA). During the incubation reaction, pNA is released from the substrate by caspase 3, which serves as a DEVDase. The release of pNA results in the production of a yellow color, which was measured by absorbance at 405 nm with a micro-plate reader (BioTek Instruments, Inc.). The amount of free pNA was calculated according to a pNA standard with a range of 0-100 μM.

3.10 Statistical analysis

Data were expressed as the mean $\pm$ standard deviation. Statistical analysis of the physiological parameters was performed using the paired t-test (SPSS version 14.0, Chicago, IL, USA). Multivariate analysis of the other parameters showed a significant interaction between two factors: the selected acupoint and the number of stimulation treatments. Therefore, comparisons were made among these groups using the analysis of variance method (ANOVA) followed by the post-hoc protected least-significant difference test. In all cases, $P<0.05$ is considered to be statistically significant.

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Chapter 4

Pre-ischemia electro-acupuncture potentiates the expression of Bcl-2 and transforming growth factor beta-1 in rat brains

4.1 Abstract

The expression of the anti-apoptotic molecules Bcl-2 and transforming growth factor beta-1 (TGFβ-1) is known to confer protective effects on the cerebral ischemia-reperfusion injury. The current study investigated the expression levels of Bcl-2 and TGFβ-1 in response to multiple pre-ischemia electro-acupuncture (EA) at acupoints ST 36 and GB 20 stimulation. Rats were divided into 5 groups: uninjured, control, non-acupoint (NA), GB 20 and ST 36. Rats in the NA, GB 20 and ST 36 groups received 30 minutes (3 times or 18 times) of EA stimulation before experimental cerebral ischemia was induced. Bcl-2 and TGFβ-1 were found to be significantly increased after pre-ischemia EA in the ST 36 groups with either 3 or 18 EA treatments ($P<0.05$). The production was higher with 18 EA treatment in the ST 36 groups ($P<0.05$). In the GB 20 groups, significant increase was only observed in TGFβ-1 with 18 EA treatment ($P<0.05$). No significant elevation of the level of TGFβ-1 was observed in th NA groups. However, the production of Bcl-2 increased with 18 treatments in the NA groups ($P<0.05$). The data suggest that multiple pre-ischemia EA at ST 36 was effective in conferring neuroprotective effect on the brain by means of up-regulation of Bcl-2 and TGFβ-1 and the effect was increase with the number of treatment.

Keywords: Cerebral ischemia; Stroke prevention; Electroacupuncture; TGFβ-1; Bcl-2; Acupoint.

4.2 Introduction

Transforming growth factor beta-1 (TGFβ-1) is a ubiquitous cytokine that influences various cell types, including microglia (Lehrmann et al., 1995) and neurons (Lehrmann et al., 1995; Henrich-Noack et al., 1996). It is virtually absent from intact brain tissue (Henrich-Noack et al., 1996; Lehrmann et al., 1998), but its expression increases strongly after trauma (Henrich-Noack et al., 1996), excitotoxic lesioning and ischemia-reperfusion injury (Wang and Wang, 1995). TGFβ-1 induction has been associated with several anti-inflammatory effects including decreased neuronal susceptibility to glutamate excitotoxicity, macrophage deactivation and astrocytic brain-derived neurotrophic factor induction (Lehrmann et al., 1998). TGFβ-1 also contributes to calcium homeostasis in nerve cells, which is important for the regulation of neuronal nitric oxide synthase (nNOS) expression (Lehrmann et al., 1995). TGFβ-1 also reduces cerebral ischemia-reperfusion induced inducible nitric oxide synthase (iNOS) expression (Thomas et al., 2001). Increased TGFβ-1 protein levels may act as an initiator of neuroprotective mechanisms. Bcl-2 is a known oncogene product that protects cells from apoptosis by preventing the release of cytochrome C (Knox et al., 1993; Ojala et al., 2000). Increased Bcl-2 expression is also neuroprotective because it decreases the formation of peroxynitrite and nitric oxide, thereby reducing infarct volume and inhibiting the activation of caspase-3 (Cao et al., 2002; Seo et al., 2002; Zhao et al., 2003).

Acupuncture, in particular electro-acupuncture (EA), has increasingly been used as a complementary therapy for pain relief (Romita et al., 1997; Tsui and Leung, 2002) and stroke rehabilitation in both Asian and western countries (Cheng, 1987; Leake and Broderick, 1998; Si et al., 1998). A considerable number of studies investigated the

effectiveness of EA therapy on patients with cerebral ischemia. Several beneficial outcomes have been noted, including reduced paralysis due to increased muscle strength (Huang et al., 1998; Xing and Zhang, 1998), improved speech (Xing and Zhang, 1998), reduced mental retardation (Xing and Zhang, 1998), restored cerebral blood flow (Tan, 1990; Zhai et al., 1993) and improved locomotion (Tan, 1990; Chang et al., 1996; Xing and Zhang, 1998; Liu et al., 1999). These observations support the hypothesis that post-ischemia EA can be an effective modality for the treatment of stroke. However, little is known about the effectiveness of pre-ischemia EA in minimizing neurological injury caused by stroke. If proven successful, pre-ischemia EA could be used as a preventive measure for patients at increased risk of stroke. In this study, we investigated the effects of multiple applications of EA stimulation before the induction of cerebral ischemia. Two acupoints, GB 20 and ST 36, were selected for this study because EA stimulation of these two points might minimize damage to brain tissue through its ability to attenuate lipid peroxidation in areas affected by stroke (Siu et al., 2004). Furthermore, GB 20 is an effective acupoint often selected for stroke rehabilitation because treatment via GB 20 greatly improves the locomotive ability of stroke patients (Tan, 1990; Liu et al., 1999). In contrast, ST 36 is a common acupoint for analgesia, spasmolysis and homeostasis (Stux and Pomeranz, 1988; Yun et al., 2002). In order to confirm the correct selection of acupoint in the tissue, the bilateral non-acupoint was selected and compared to the selected acupoint. After the induction of cerebral ischemia, the effects of EA stimulation at different acupoints were assessed by measuring the expression of TGFβ-1 and Bcl-2. As described above, the increased production of TGFβ-1 and Bcl-2 has been shown to be neuroprotective against cerebral ischemia.

4.3 Materials and methods

4.31 Experimental design

All experimental procedures were approved by the Hong Kong Polytechnic University animal ethical committee. A total of 68 male Sprague-Dawley rats weighing 300-350 g were used. Firstly, twelve rats were used to measure common physiological parameters in blood obtained from the femoral artery 15 minutes before and during cerebral ischemia. Then eight rats were assigned to the uninjured group for measurement of the parameters listed below. Forty-eight rats were evenly divided into four experimental groups: a control group (rats with general anesthesia without EA), a non-acupoint (NA) group (rats with EA stimulation at non-acupoints), a GB 20 group (rats with EA stimulation at GB 20), and an ST 36 group (rats with EA stimulation at ST 36). EA stimulation was carried out on alternate days before the induction of cerebral ischemia. In each group, six rats were subjected to 3 EA treatments conducted for a week and another six rats were subjected to 18 EA treatments conducted over 6 consecutive weeks. The rats in the experimental groups were then subjected to transient focal cerebral ischemia followed by harvesting of their brains on post-ischemia day 4.

4.32 Pre-ischemia electro-acupuncture (EA)

A 30-minute EA treatment was applied by an acupunctoscope device (Model G6805-2, Smeif, Shanghai, China) (Voltage: 0.7 V, frequency: 2 Hz, duration: 0.5 ms) as previously described (Siu et al., 2004). In the GB 20 and ST 36 groups, EA was applied at corresponding bilateral points. GB 20 is anatomically located on the posterior aspect of

the neck, below the occipital bone, in the depression between the sternocleidomastoid muscle and the trapezius muscle (Ellis et al., 1991). ST 36 is anatomically located near the knee joint of the hind limb 2 mm lateral to the anterior tubercle of the tibia (Ellis et al., 1991). In the NA group, EA was applied at a non-acupoint located midway between the coccyx and the hip joint. The results from the use of non-acupoints for control purposes highlight the significance of selecting the correct acupoint. Apart from electrical stimulation, needle puncture is also one of the acupoint stimulation methods, so it is not appropriate to serve as a control. Previous studies reported that treatment of acupuncture at ST 36 could alleviate ischemia-induced apoptosis (Chung et al., 2007), inhibit inflammation (Chae et al., 2007) and promote neurogenesis (Hwang et al., 2010).

4.33 Induction of transient focal cerebral ischemia

Transient focal cerebral ischemia was induced by the occlusion of the right middle cerebral artery (MCA) for 1 hour. Rats were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg, Alfasan, Holland) and xylazine (7 mg/kg, Alfasan, Holland). The temporalis muscle was then briefly separated in the plane of its fiber bundles to expose the zygoma and squamosal bones. A 5-mm x 5-mm burr hole was made to expose the MCA, which was then occluded for 1 hour followed by reperfusion. During the surgical procedure, the rectal temperature of the rats was monitored and maintained at about 37 °C with an overhead lamp. After recovery from the experimental surgery, all rats were allowed to recover at ambient temperature (21-23 ºC) with food and water made available *ad libitum* until harvest. To measure physiological parameters, arterial blood samples were taken from the femoral artery 15 minutes before and after MCA occlusion for the determination of pH, arterial partial pressure of oxygen (paO_2) and carbon dioxide ($paCO_2$)

by a Blood Gas and Electrolyte System (Radiometer ABL505, Copenhagen, Denmark).

4.34 Chemicals and antibodies

Unless otherwise stated, all materials were of analytical grade and obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Primary antibodies (200 μg/ml) against TGFβ-1 and Bcl-2 were used at a dilution of 1:200. Beta-actin (β-actin) was employed as an internal control at a dilution of 1:2000. Secondary antibodies conjugated with horseradish peroxidase directed against rabbit immunoglobulin G were obtained from Pierce Chemical Co. (Rockford, IL, USA) and used at a dilution of 1:1000. Protein concentrations were determined with a protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

4.35 Sample collection

Rats were sacrificed 4 days after ischemia-reperfusion with an overdose ketamine and xylazine mixture. Transcardial perfusion was performed with 0.38 % sodium citrate in normal saline for 10 minutes followed by 0.9 % sodium chloride for 5 minutes. The harvested right hemisphere of the brain was weighted and homogenized in 20 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 3000 g for 10 minutes at 4 °C and the supernatant was collected for analysis.

4.36 Expression of TGFβ-1 and Bcl-2

TGFβ-1 and Bcl-2 were quantitated by western blots probed with specific primary antibodies. A 15% SDS-PAGE (SDS-polyacryamide gel electrophoresis) resolving gel was

prepared for western blotting for the detection of TGFβ-1 and Bcl-2. In brief, protein homogenate (200 μg) resolved by gel electrophoresis was electroblotted to nitrocellulose membranes at a constant voltage of 25 V for 16 hr. After transfer of the proteins, the membranes were blocked with skim milk in 1X Tris-Buffered Saline and Tween 20 (TBS-T) solution, and they were then probed with primary antibodies raised against TGFβ-1 and Bcl-2 overnight at 4 ºC with gentle shaking. Subsequently, the probed blots were incubated with secondary antibodies with gentle shaking. The immunolabeled proteins were detected by their reaction with a chemiluminescent substrate (Pierce Chemical Co., Rockford, IL, USA). The chemiluminescence released was captured and quantified. Selected blots were re-probed with β-actin as an internal control.

4.37 Statistical analysis

Data were expressed as the mean ± standard deviation. Statistical analysis of the physiological parameters was performed using the paired t-test (SPSS version 14.0, Chicago, IL, USA). Multivariate analysis of the other parameters showed a significant interaction between two factors: the selected acupoint and the number of pre-ischemic EA stimulation treatments. Therefore, comparisons were made among these groups using the analysis of variance method (ANOVA) followed by the post-hoc protected least-significant difference test. In all cases, $P<0.05$ is considered to be statistically significant.

4.4 RESULTS

4.41 Physiological parameters

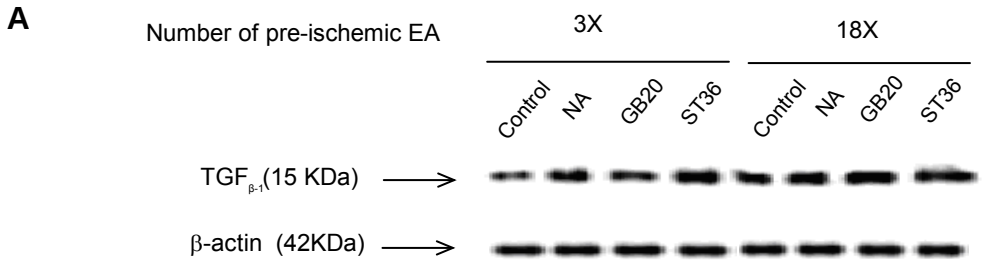
The physiological data obtained before and during transient focal cerebral ischemia are shown in Table 4.1. The values of the parameters measured were consistent with previously reported values (Leker et al., 2001; Siu et al., 2004). The difference between the values of paO_2 measured before (104.75 ± 10.76 mmHg) and after (107.80 ± 6.67 mmHg) ischemia was not statistically significant (t=0.846, degrees of freedom (d.f.) =5, a=0.05). Similarly, the values of $paCO_2$ measured before ischemia, 43.45 ± 3.5 mmHg, was comparable to that after MCA occlusion, 44.30 ± 5.06 mmHg (t=0.270, d.f.=5, a=0.05). In addition, there was no significant difference between the values of blood pH measured before (7.229 ± 0.029) vs. after (7.223 ± 0.039) cerebral ischemia. These findings suggest that the observed changes in cerebral tissues were not a result of physiological changes.

	paO_2 (mmHg)	$paCO_2$ (mmHg)	pH (units)
Pre-ischemia (n= 6)	104.75 ± 10.76	43.45 ± 3.5	7.229 ± 0.029
Post-ischemia (n= 6)	107.8 ± 6.67	44.30 ± 5.06	7.223 ± 0.039

Table 4.1 Physiological data from rats subjected to transient focal cerebral ischemia. Data (mean ± standard deviation) obtained from arterial blood at 15 min before and after MCA occlusion. Abbreviations: paO_2, partial oxygen pressure; $paCO_2$, partial carbon dioxide pressure.

4.42 Expression of TGFβ-1

The expression of TGFβ-1 in the different groups is shown in Figure 4.1. TGFβ-1 was not detected in the uninjured group. After 3 applications of EA stimulation, no significant differences were observed between the control, NA and GB 20 groups. However, TGFβ-1 expression was significantly higher in the ST 36 group (4.44 ± 1.18 pg TGFβ-1 per mg protein; $P<0.05$) when compared to the other three groups. Similarly, after 18 applications of EA stimulation, the highest level of TGFβ-1 (6.72 ± 1.99 pg TGFβ-1 per mg protein) was detected in the ST 36 group. This expression level was significantly higher than those of the other three groups ($P<0.05$). In addition, the GB 20 group with the same treatment frequency also produced two-fold more TGFβ-1 than the control group ($P<0.05$). Nevertheless, an increase in TGFβ-1 expression associated with a greater number of treatments was observed in the ST 36 group in which 18 EA applications induced about 50% more TGFβ-1 than did 3 EA applications ($P<0.05$).



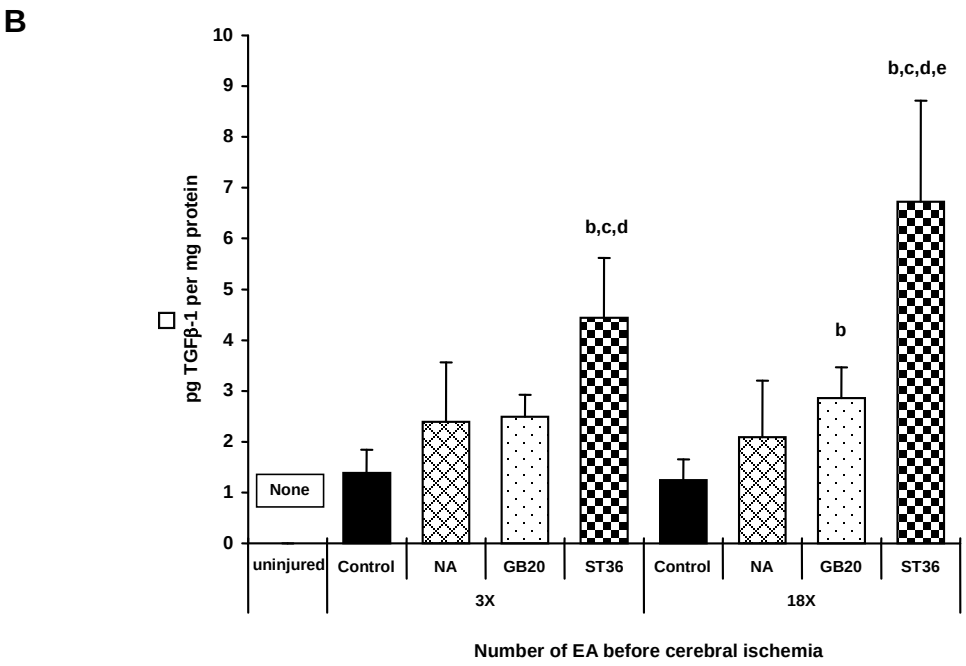


Figure 4.1 Expression of transforming growth factor beta-1 (TGFβ-1): (A) Selected western blot specific for TGFβ-1 in the experimental groups with β-actin as an internal control. (B) Quantification of TGFβ-1 (mean ± standard deviation) in each experimental group (n=6) and in the uninjured group (n=8) at post-injury day 4. The unit is expressed as pg expressed TGFβ-1 per mg protein. Under the same treatment time, the letters (b, c, d) indicate significant differences ($P<0.05$) in comparison to the control group (b) and to the NA group (c) and between the GB 20 and ST 36 groups (d). The letter e indicates a significant difference between a group with 18 EA stimulations and the corresponding group with 3 EA stimulations.

4.43 Expression of Bcl-2

The expression of Bcl-2 in the different groups is shown in Figure 4.2. The amount of Bcl-2 expressed in the uninjured group was 0.502 ± 0.06 pg Bcl-2 per mg protein. With 3

applications of EA stimulation, a significant increase in Bcl-2 expression was only observed in the ST 36 group (P<0.05). Bcl-2 expression was higher with 18 applications of EA stimulation than with 3 applications in all groups. Relative to the control and GB 20 groups, the ST 36 group expressed 29% and 15% (respectively) more Bcl-2 (P<0.05). However, Bcl-2 expression was also upregulated in the NA group (18-times), similar to the expression in the ST36 group.

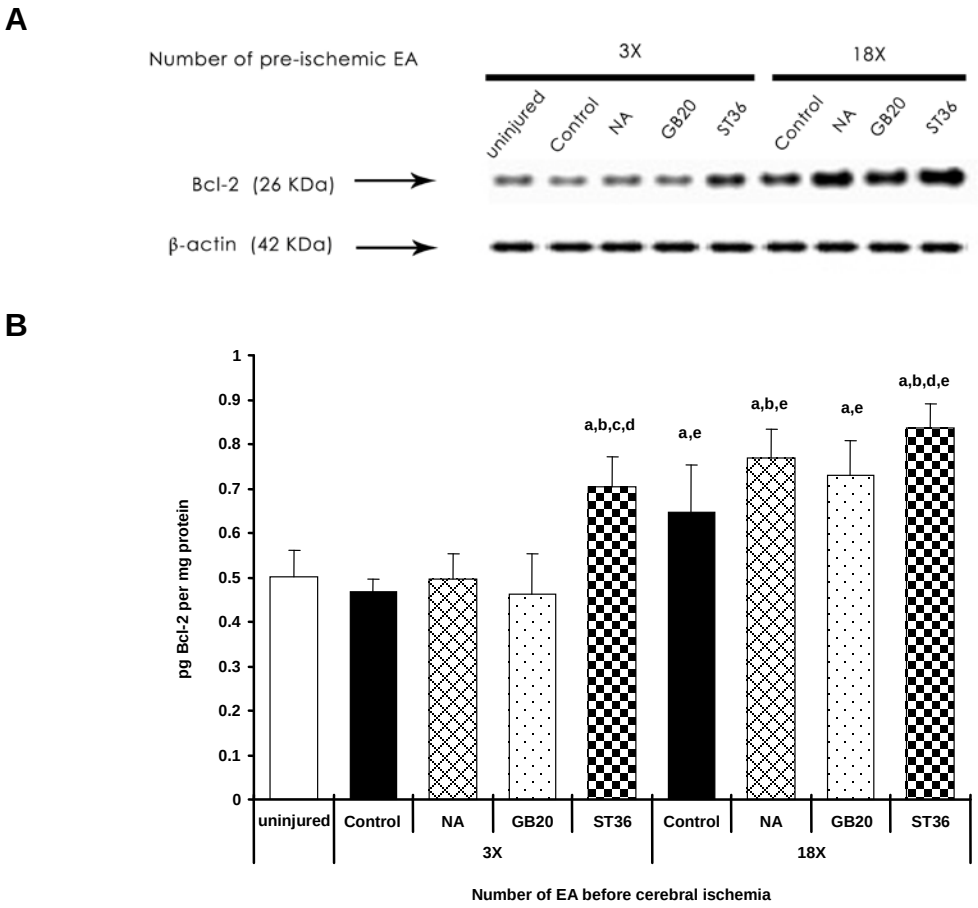


Figure 4.2 Expression of Bcl-2: (A) Selected western blot specific for Bcl-2 in the experimental groups with β-actin as an internal control. (B) Quantification of Bcl-2 (mean ± standard deviation) in each experimental group (n=6) and in the uninjured group (n=8) at post-injury day 4. The unit is expressed as pg expressed Bcl-2 per mg protein. The letter (a) revealed a significant difference between the uninjured group and each experimental

group ($P<0.05$). For the same treatment time, the letters (b, c, d) indicate significant differences ($P<0.05$) in comparison to the control group (b) and to the NA group (c) and between the GB 20 and ST 36 groups (d). The letter e indicates a significant difference between a group with 18 EA stimulations and the corresponding group with 3 EA stimulations.

4.5 DISCUSSION

The current study investigated the effect of pre-ischemia EA on the expression of TGFβ-1 and Bcl-2 at day 4 post-injury. It has been previously reported that TGFβ-1 mRNA expression increases and reaches a maximum at day 7 after transient and permanent occlusion of MCA (Lehrmann et al., 1998; Yamashita et al., 1999). This is why post-injury day 4 was selected in the present study for investigation. In the present study, the expression of TGFβ-1 was not detected in the uninjured group. Similar findings were also reported in the expression of TGFβ-1 mRNA in unoperated rats (Lehrmann et al., 1995). After a 1-hour occlusion of MCA, a significant increase in the level of TGFβ-1 protein in the ST 36 group (3-times EA and 18-times EA) and the GB 20 group (18-times EA) was observed. The administration of TGFβ-1 has been reported to decrease infarct size after focal cerebral ischemia in rats (Prehn et al., 1993; McNeill et al., 1994) and rabbits (Gross et al., 1993), and to protect hippocampal neurons after global cerebral ischemia (Henrich-Noack et al., 1996). Therefore, pre-ischemia EA may have a beneficial effect in neuroprotection after cerebral ischemia by upregulating the content of TGFβ-1. The neuroprotection may take place in penumbral region of the cerebral cortex and striatum where the elevated expression of TGFβ-1 messenger ribonucleic acid (m-RNA) following occlusion of MCA was reported recently (Vincze et al., 2010).

Bcl-2 also plays an important role in neuroprotection in cerebral ischemia. Decreased infarct area was observed in over-expressed Bcl-2 transgenic mice (Martinou et al., 1994), whereas increased infarct area was found in Bcl-2 knockout mice (Hata et al., 1999). The neuronal protection controlled by Bcl-2 is through the inhibition of free radical production (Lee et al., 2001), cytosolic accumulation of cytochrome c and caspase-3 activation (Zhao et al., 2003). In the current study, after pre-ischemia EA, the level of Bcl-2 was elevated at ST 36 (3-times and 18-times) but not at GB 20. In contrast of our previous study, pre-ischemia EA at GB 20 showed that malondialdehyde production was inhibited (Siu et al., 2004) suggesting that different acupoints may exert neuronal protection through different biochemical pathways. Hippocampal CA1 region was most frequently employed for Bcl-2 investigation under cerebral ischemic condition due to its vulnerability to cerebral injury. Ferrer et al. (1998) observed a marked increase of Bcl-2 expression in hippocampal CA1 region at post-injury day 4. Zhang and Wang (1999) also reported that Bcl-2 mRNA was up-regulated in hippocampus following cerebral ischemia. In a recent study, EA at ST 36 and GV 20 were able to increase Bcl-2 expression in hippocampal CA1 area (Bu et al., 2010).

In both production profiles of TGFβ-1 and Bcl-2, the up-regulation was higher in 18-times application when compared with those after 3 times at pre-ischemia EA at ST 36. More benefits might be derived from a higher number of pre-conditioning EA sessions. However, the current study employed only two time-points, which might not be able to determine if and when any plateau effect or adverse effect may be achieved.

Apart from the current beneficial effect of pre-ischemia EA on cerebral ischemia, previous studies also revealed that reduced infarct size, neurological deficit and apoptosis were

observed even in a single 30-minutes pre-ischemia EA at acupoint GB 20 through the regulation of endocannabinoid system (Wang et al., 2009). Dong et al. (2009) also demonstrated that repeated EA preconditioning (5 days) at acupoint GB 20 could lower the infarct size and neurological deficit score with the inhibition of matrix metalloproteinase-9 expression and activity. Our research team also reported in a previous study that multiple pre-ischemia EA at ST 36 and GB 20 could reduce the production of malonadialdehyde (Siu et al., 2004).

Although previous studies have focused on stroke rehabilitation by EA stimulation, the present study explored another clinical application of EA stimulation. Pre-ischemic EA stimulation is particularly important for specific high-risk groups, such as patients with high blood pressure or other congenital characteristics that predispose them to stroke. Applying EA stimulation at either GB 20 or ST 36 on alternate days each week might ameliorate the severity of brain damage after a stroke and shorten the time of recovery.

4.6 Conclusion

Data from this study confirmed that multiple pre-injury EA stimulations at ST 36 could effectively increase the production of both TGFβ-1 and Bcl-2, with an up-regulation of TGFβ-1 correlated to a neuroprotective effect at GB 20. These proteins work together to reduce the extent of brain tissue damage after cerebral ischemia-reperfusion. EA treatment can be used as a preventive measure against stroke damage. In addition, TGFβ-1 promotes the neuroprotection of proteins against expected oxidative stress by regulating the apoptotic cascade. The small sample size of this study is an obvious

limitation; further experiments with a larger number of animals are required for a more detailed evaluation of apoptotic events.

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Chapter 5

The effect of pre-ischemia acupoint high voltage electrical stimulation on the expression of iNOS and MDA following experimental cerebral ischemia in rat model

5. 1 Abstract

Aims: Different forms of acupoint stimulation were applied in pre-ischemia measure. Clinical application of post-ischemia acupoint high voltage electrical stimulation (HVES) is popular but its pre-ischemia contribution and possible cellular mechanism remain unknown. The current study investigated the effect of pre-ischemia acupoint HVES on minimizing the cerebral neuronal damage.

Main methods: Animals were divided into four groups: normal, ischemia, 5-times and 18-times treatments. All the subjects in treatment groups received corresponding pre-ischemia acupoint HVES for 30 minutes in alternative day at different acupoints bilaterally: ST 36, LI 10 or ST 36 with LI 10.

Key findings: The inducible nitric oxide synthase (iNOS) and malondialdehyde (MDA) of brain homogenate were investigated. The production of iNOS was significantly reduced in 5-times at ST 36, 5-times at ST 36 and LI 10 and 18-times at ST 36 groups ($p<0.05$). The significant decrease of the concentration of MDA was observed in 5-times at ST 36 and 18-times at ST 36 groups ($p<0.05$). No significant difference was observed in the same acupoint of 5-times and 18-times stimulation.

Significance: Pre-ischemia acupoint HVES could attenuate the production of iNOS and MDA. However, the protective effect is acupoint-specific. Positive outcome occurred at

ST 36, but not at LI 10. Bilateral single acupoint stimulation showed the more favourable effect than multi-point did. Short-term stimulation demonstrated similar beneficial result as the long-term did.

Key words: lipid peroxidation, malondialdehye, inducible nitric oxide synthase

5.2 Introduction

As one of the leading causes of death and serious debilitation, many studies investigated pre-ischemia measure to minimize neuronal damage following cerebral ischemia. Apart from those life-style changing strategies, pre-ischemia acupoint stimulation was also employed in reducing cerebral ischemic damage. Xiong et al. (2003) investigated the preconditioning effect of electroacupuncture (EA) at acupoint GV 20 on cerebral ischemic rat. The subjects received 30-minutes pre-ischemia stimulation for 5 days. Both infarct lesion and neurological deficit score were reduced in the treatment group. One year later, our research team tried to find out the possible mechanism of pre-ischemia EA on cerebral ischemia (Siu et al., 2004a). We applied 30-minutes pre-ischemia stimulation at acupoints ST 36 and GB 20 for 3 days and 18 days. The protective effect was demonstrated by the attenuation of the production of malonadialdehyde (MDA) of the brain homogenate. Dong et al. (2009) also demonstrated that repeated EA preconditioning (5 days) at acupoint GB 20 could lower the infarct size and neurological deficit score with the inhibition of matrix metalloproteinase-9 expression and activity.

Clinical treatment of acupoint high voltage electrical stimulation (HVES) following cerebral stroke was found to be effective in improving hemiplegic patients' motor function (Wu 1994; Zhang ad Fan 1998; Zhao et al., 2006) and facial paralysis (Yu and Li, 1994). Although there were a lot of beneficial effects reported by post-ischemia acupoint HVES after stroke, it remains uncertain if acupoint HVES can be used as a pre-stroke preventive measure to minimize the cerebral damage.

It is well known that nitric oxide is formed during cerebral ischemia to exert protective or

harmful effect depending on which nitric oxide synthase involved. Inducible nitric oxide synthase (iNOS) mRNA, protein and enzyme activity were expressed in the brain after transient or permanent ischemia in rodents (Grandati et al., 1997; Iadecola et al., 1995b) and in human cerebral infarcts (Forster et al., 1999). Inhibition of iNOS activity ameliorates cerebral ischemic damage by decreasing of glutamate release and cerebral infarct (Iadecola et al., 1995a; Pérez-Asensio et al., 2005) and increasing of the survival of hippocampal pyramidal neurons (Danielisová et al., 2004).

During cerebral ischemia, a large amount of free radical is produced. Lipid peroxidation is resulted from the loss of electrons of lipids in cell membrane to the free radical. The consequence of this oxidative degradation is cell damage. Lipid peroxidation is therefore regarded as one of the major causes of post-ischemic injury (Yoshida et al., 1980). MDA level is a widely accepted markers and a by-product of lipid peroxidation. Significantly higher level of MDA was found in cerebral ischemic rat (Bromont et al., 1989). In human, functional score of stroke patients was found to be negatively correlated with serum MDA level (Polidori et al., 2002; Ozkul et al., 2007).

The current study used iNOS and MDA as the markers to demonstrate the effect of pre-ischemia acupoint HVES on the production of free radicals and its consequence, lipid peroxidation, after experimental induced cerebral ischemia in rat model.

5.3 Materials and methods

5.31 Grouping of animal

A total of 40 Sprague-Dawley rats (male, 330g - 350g) were used under the procedure for the care of laboratory animals lay by The Hong Kong Polytechnic University. Rats were divided into 4 groups: normal (n=5), Ischemia (n=5), 5-times stimulation (n=15) and 18-times stimulation (n=15). In normal group, rats received neither treatment nor operation. In ischemia group, rats were anesthetized and induced cerebral ischemia. In 5-times stimulation group, rats were received 5-times bilateral acupoint HVES at ST 36 (n=5) or at LI 10 (n=5) or at both ST 36 and LI 10 (n=5) in 2 weeks before the induction of cerebral ischemia. In 18-times stimulation group, same stimulation protocol was performed in 6 weeks before the induction of cerebral ischemia.

5.32 Pre-ischemia acupoint high voltage electrical stimulation (HVES)

Pulsed pre-ischemia acupoint HVES (0.4ms) was delivered bilaterally at corresponding acupoint through a pair of fixed round-shaped electrodes measuring 2mm in diameter for 30 minutes (Model ZDL-4, Nanjing, China) at voltage 1,000 V with frequency 2.5 Hz. Intensity was set at the level of muscle contraction just initiated. Ultrasound gel was applied for better conductivity (Chattanooga Group, Modialloc Victoria Australia). Treatment was taken corresponding to the treatment group with frequency of 3 times per week before the induction of cerebral ischemia.

5.33 Induction of transient focal cerebral ischemia

Twenty-four hours after the last treatment, transient focal cerebral ischemia was induced by temporarily occluding the right middle cerebral artery with surgical clip for 1 hour (Leung et al., 2002; Siu et al., 2004a, 2004b, 2005). Rats were anesthetized by intraperitoneal injection of ketamine (70 mg/kg, Alfasan, Holland) and xylazine (7mg/kg, Alfasan, Holland). After a 2 cm incision of scalp was made, the temporalis muscle was separated in the plane of its fiber bundles to expose the zygoma and squamosal bones. At the anterior junction of the zygoma and squamosal bones, a 5 mm x 5 mm burr hole was made using a saline-cooled electric drill. The exposed middle cerebral artery was surgically clipped for 1 hour and then released for reperfusion.

5.34 Sample collection

Rats were sacrificed 4 days after ischemia-reperfusion with an overdose ketamine and xylazine mixture. The sampling point was determined by considering the maximum nitric oxide synthase (NOS) activity after the induction of cerebral ischemia (Leung et al. 2002). Transcardial perfusion was performed with 0.38 % sodium citrate in normal saline for 10 minutes followed by 0.9 % sodium chloride for 5 minutes. The harvested right hemisphere of the brain was weighted and homogenized in 20 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 3000 g for 10 minutes at 4 ^{o}C and the supernatant was collected for analysis.

5.35 Inducible nitric oxide synthase expression

Protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins in the solubilized homogenate (100 µg each) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10 % acrylamide separating slab gel with 5 % acrylamide stacking gel. After electrophoresis, the separating gels were electroblotted overnight onto nitrocellulose membranes at 4 °C. The membranes were incubated overnight at 4 °C with rabbit antibodies against rat iNOS (1:200 dilution, 0.5 µg/ml, Santa Cruz Biotechnology, Inc.). After reacting with Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL, USA), chemiluminescence was captured by the UVP-Chemi System (UVP, Inc., Upland, CA, USA), and signal intensities (expressed as ng/mg total protein) were analyzed using Lab-Works software (UVP, Inc., Upland, CA, USA). Selected blots were directly reprobed with anti-β-actin antibody (1:5000; Sigma Chemical Co., St. Louis, MO, USA), and the resulting signal intensity was used to confirm equal sample loading and even transfer. The expression of apoptotic parameters is defined as ng/mg protein.

5.36 Lipid peroxidation assay

Lipid peroxidation and oxidative stress at cellular level was determined by titrating the brain tissue of the end-product, malondialdehye (MDA), using an immunoassay kit (Oxis International, Inc., Portland, OR, USA), which reflected the formation of a chromophore with MDA molecule (Erdelmeier et al., 1998). Each sample mixture was composed of 50 µl of supernatant, 50 µl of 20 mM Tris-HCl (pH 7.4) and 325 µl of 10.3 mM N-methyl-2-phenylindole in acetonitrile. To assay for the quantity of MDA, sample mixture was added with 75 µl of 15.4 M methanesulfonic acid and incubated at 45 °C for 40 minutes. After

incubation, samples were cooled on ice and their absorbance was read at 586 nm. The unit was defined as μM per mg homogenate protein.

5.37 Data analysis

Data was analyzed using one-way ANOVA followed by post-hoc Least Significant Difference test (SPSS v.17, SPSS Inc.). The statistical significance was defined as $p<0.05$. Results were given as mean + standard deviation. The main effects and interaction of two factors, between-subject factors (treatment-induction to treatment-normal) and within-subject factors (treatment sessions), were investigated.

5.4 Results

5.41 Inducible nitric oxide synthase (iNOS)

In Figure 5.1, when compared to the normal group, the production of iNOS were significantly up-regulated in all groups except 5-times HVES at ST 36 ischemia and 18-times HVES at ST 36 groups ($p<0.05$). Nitric oxide production was considered to take place following experimentally-induced cerebral ischemia.

When compared to the ischemia group, the level was significantly reduced in 5-times HVES at ST 36 ischemia, 5-times HVES at ST 36 and LI 10 ischemia and 18-times HVES at ST 36 groups ($p<0.05$). Those pre-treatment protocols were able to reduce the expression of iNOS in cerebral ischemia condition.

When compared 5-times and 18-times stimulation, no corresponding groups demonstrated a significant change. The number of stimulation could provide similar result in the post-ischemia iNOS production.

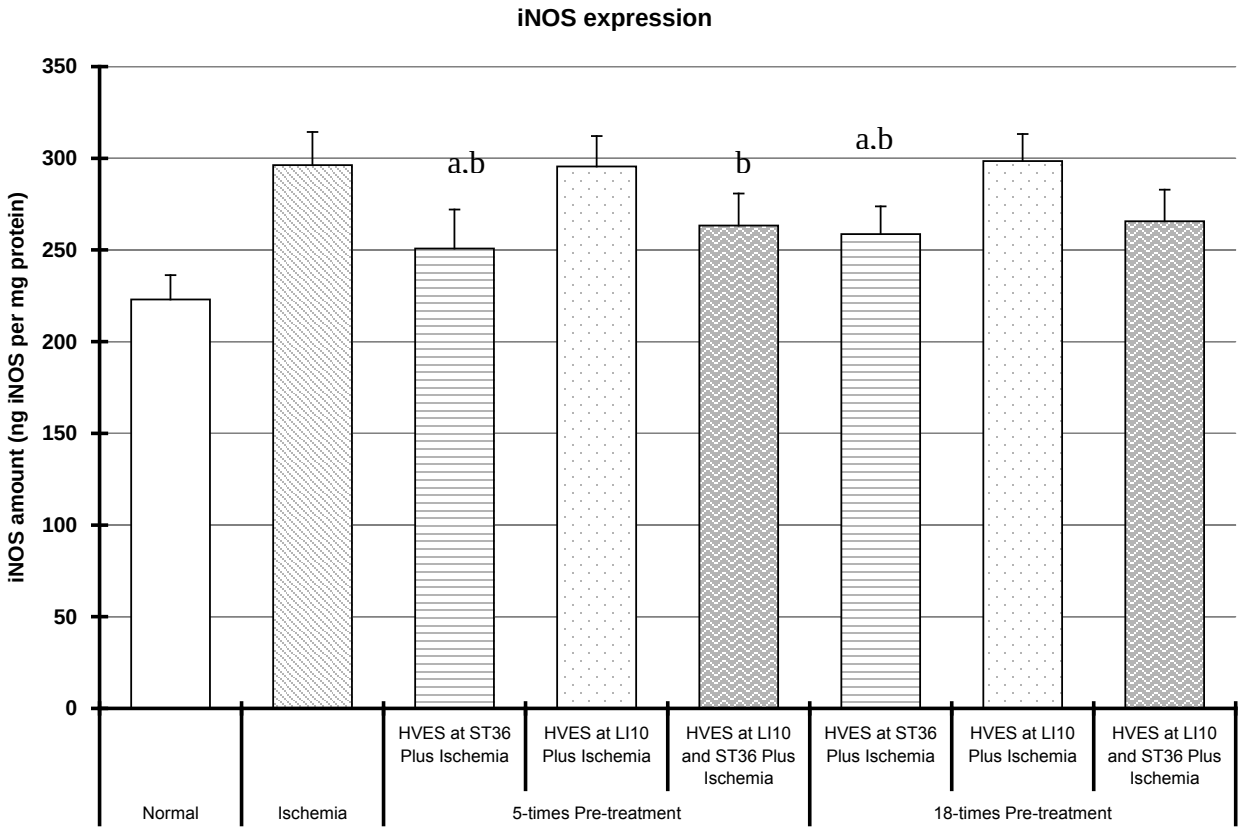

Figure 5.1 Production of iNOS of different groups after multiple pre-ischemia acupoint HVES. Data was represented as mean + standard deviation. Symbols a and b represented the significance difference to normal group ($p < 0.05$) and to the ischemia group ($p < 0.05$) respectively.

5.42 Lipid peroxidation

In Figure 5.2, when compared to the normal group, the production of MDA was significantly greater in all groups except 5-times HVES at ST 36 ischemia and 18-times

HVES at ST 36 groups ($p<0.05$). This implied lipid peroxidation was taken place after experimentally-induced cerebral ischemia.

When compared to the ischemia group, both 5-times HVES at ST 36 ischemia and 18-times HVES at ST 36 groups were significantly reduced the production of MDA in cerebral ischemia condition ($p<0.05$).

Same as the result of the expression of iNOS, no significant difference was observed when compared the corresponding groups of 5-times and 18-times stimulation.

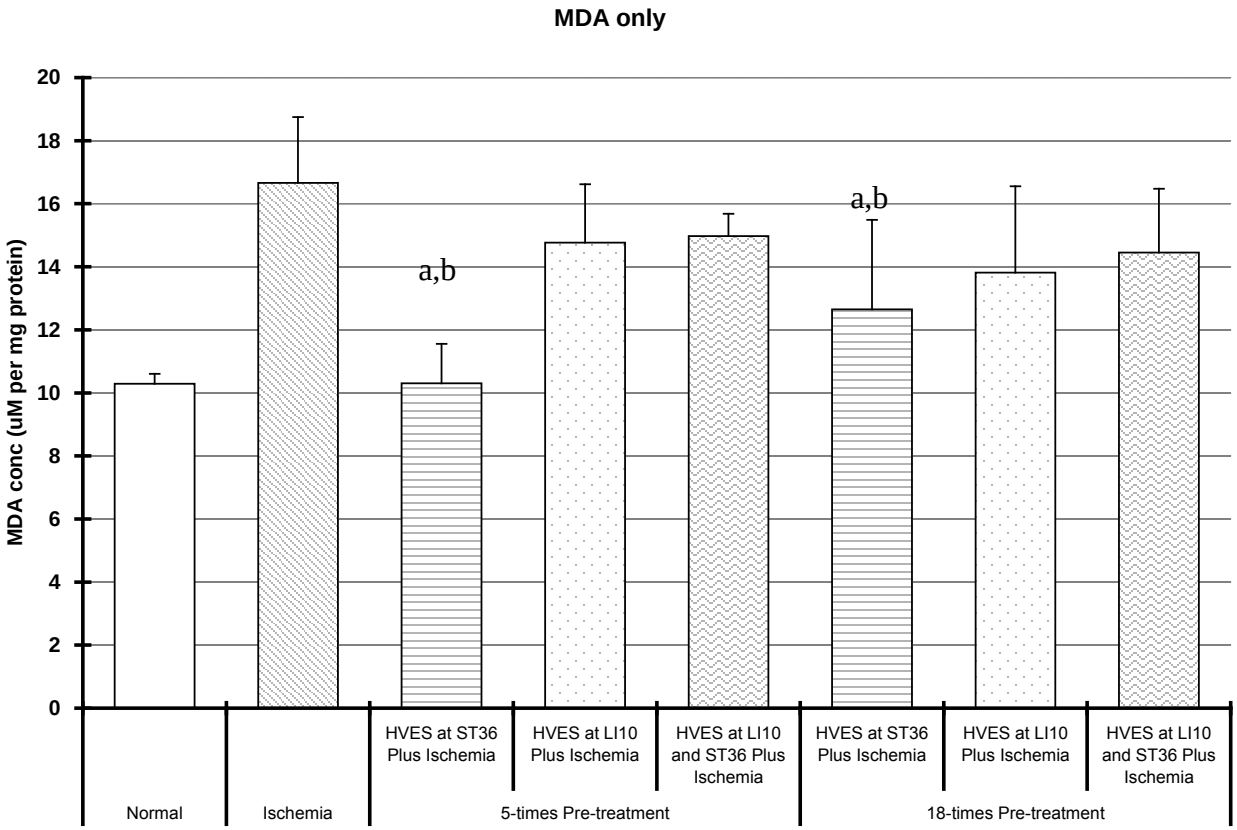

Figure 5.2 Production of MDA of different groups after multiple pre-ischemia acupoint HVES. Data was represented as mean + standard deviation. Symbols a and b represented the significance difference to normal group ($p< 0.05$) and to the ischemia

group ($p < 0.05$) respectively.

5.5 Discussion

The present study provided evidence of the efficacy of pre-ischemia acupoint HVES on cerebral ischemia by minimizing the neuronal damage through the reduction of the free radical synthesizing enzyme.

5.51 Single acupoint application at ST 36/LI 10

Inducible nitric oxide synthase (iNOS) is responsible for cytotoxicity through the production of large amounts of nitric oxide (NO). iNOS inhibitor was found to reduce infarct size in neocortex in comparison with vehicle-treated controls (Iadecola et. al 1996). In our present study, both 5-times and 18-times pre-ischemia acupoint HVES at ST 36 could reduce the production of iNOS and MDA. The possible mechanism may be hindering the production of NO to down-regulate the end product of lipid peroxidation, MDA. No similar beneficial outcome was observed at LI 10 despite that ST 36, LI 10, or pair of acupoints was clinically used in patients with stroke. Although previous report even demonstrated a convalescent curative effect of post-ischemia by choosing LI 10 on affected upper limb (Zhou et al., 2009), the current apparent contradicted result may draw more attention to the selection of acupoint in pre-ischemia application from cellular point of view. This acupoint-specific characteristic was in concert with our previous pre-ischemia study of EA (Siu et al., 2004a) which also indicated that the selection of non-acupoint was not only non-beneficial but harmful in terms of the production of MDA. The result of infarct size and functional improvement is worth further investigation to show if cellular change can extend to the

physical aspect.

5.52 Combined acupoint application at ST 36 and LI 10

Combined application of acupoints is popular in most of the acupunctural therapy. However, in our study, this pairing effect (ST 36 and LI 10) did not provide further improvement in the reduction of iNOS and MDA. We first believed that combination use of acupoints may give extensive beneficial effect. However, only 5-time stimulation of ST 36 and LI 10 significantly reduced the production of iNOS. The same group of stimulation could not lead to change in the reduction of MDA. We assumed the unfavourable outcome was resulted from the selection of LI 10. This is valuable to further investigate the combination effect of acupoint by selecting another acupoint later.

5.53 Duration of pre-ischemia treatment

The current result showed that the longer pre-treatment of acupoint HVES did not provide further protection. Both the levels of iNOS and MDA in 5-times and 18-times stimulation of ST 36 did not showed significant difference to each other. It seems that pre-ischemia acupoint HVES demonstrated the therapeutic pattern as our previous pre-ischemia EA study (Siu et. al 2004a). A recent study even demonstrated that reduced infarct size, neurological deficit and apoptosis were observed in a single 30-minutes pre-ischemia EA at acupoint GB 20 through the regulation of endocannabinoid system (Wang et al., 2009). Suggestion to high risk group of stroke patient to undergo HVES is therefore enough to protect neuronal cells from damage.

5.6 Conclusion

The results of the present study provided evidence of the efficacy and the possible protective mechanism of pre-ischemia acupoint HVES on cerebral ischemia. The beneficial role of acupoint HVES was observed in both 5-times and 18-times application at ST 36 in decreasing the production of iNOS and MDA. Since the number of stimulation at ST 36 did not demonstrated significant difference, further investigation is needed to explore this observation in pre-ischemia acupoint HVES.

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Chapter 6

The effect of low-energy laser irradiation on apoptotic factors following experimentally induced transient cerebral ischemia

6.1 Abstract

Apoptosis, or programmed cell death, resulting from cerebral ischemia may be related to decreased levels of anti-apoptotic factors, such as Akt, pAkt, pBAD and Bcl-2, and increased levels of pro-apoptotic factors, such as BAD, caspase 9 and caspase 3 activity. In this study, we investigated the effects of low-energy laser (660 nm) irradiation (LLI) on the levels and activity of various anti- and pro-apoptotic factors following ischemia. Transient cerebral ischemia was induced in Sprague–Dawley rats by unilateral occlusion of the middle cerebral artery for 1 hour, followed by reperfusion. LLI was then directed on the cerebrum for varying lengths of duration (1, 5 or 10 min at an energy density of 2.64 J/cm^2, 13.2 J/cm^2 and 24.6 J/cm^2, respectively). The expression levels of Akt, pAkt, BAD, pBAD, Bcl-2, caspase 9 and caspase 3 activity were measured 4 days post-injury. The levels of Akt, pAkt, Bcl-2 and pBAD were significantly increased following laser irradiation. In addition, LLI significantly decreased caspase 9 and caspase 3 activity caused by ischemia-reperfusion. LLI may protect the brain by up-regulating Akt, pAkt, pBAD and Bcl-2 expression and down-regulating caspase 9 and caspase 3 expression following transient cerebral ischemia. This modality is a promising protective therapeutic intervention after strokes or other ischemic events.

Keywords: laser, stroke, caspase, apoptosis, ischemia

6.2 Introduction

Apoptosis is an active form of cell death and is morphologically characterized by cell shrinkage, organelle re-localization, chromatin condensation and the production of apoptotic bodies (Kerr et al., 1972; Yao et al., 2001). Apoptosis may serve as a prominent form of neuronal death in strokes. Cell survival is controlled by an intricate balance between the signals promoting survival and the signals promoting death. The mitochondrial apoptotic cascade is initiated by the cleavage of procaspase 9 by apoptotic protease activating factor 1 and the cytochrome c complex, which then triggers sequential activation of the caspase 3 cascade (Ouyang et al., 1999). Cell survival is promoted when the serine/threonine kinase (Akt) and its active phosphorylated form (pAkt) inhibit pro-apoptotic signals, including caspase 9 and BAD (the forkhead family of transcription factors whose phosphorylated form (pBAD) is believed to be inactive and therefore anti-apoptotic) (Datta et al., 1999). In addition, Bcl-2, an oncogene product, protects cells from apoptosis by preventing the release of cytochrome c (Adams and Cory, 1998; Knox et al., 1993; Ojala et al., 2000).

Low-energy laser irradiation (LLI) has been shown to be effective in cerebral ischemia. Lapchak et al. (2004) showed that LLI (808 nm, 7.5 mW/cm^2 or 25 mW/cm^2) improves clinical rating scores in a rabbit embolic stroke model. Oron and colleagues (2006) applied LLI (808 nm, 7.5 mW/cm^2) on both the occlusion and insertion stroke models in rats and found that neurological deficits were significantly reduced. In clinical studies, Lampl et al. (2007) applied LLI (808 nm, 10 mW/cm^2) on 120 ischemic stroke patients within 24 hours from the onset of stroke. Both the National Institutes of Health Stroke Scale (NIHSS) and the modified Ranki Scale (mRS) scores were significantly lowered in treated groups.

However, only patients with NIHSS 7-15 benefited from LLI of a nearly identical protocol (Zivin et al., 2009). Currently, a large clinical trial is ongoing with an enrollment of 1000 patients with a pre-determined NIHSS baseline between 7 and 17. Apart from the application in cerebral stroke, the infarct size was also reduced by LLI (804 nm, 38 mW) in rat-myocardial infarction model (Yaakobi et al., 2001). A number of studies tried to investigate the protective mechanism of LLI. Shefer et al. (2002) demonstrated the protective ability of LLI (632 nm, 4.5 mW) through the promotion of the survival of muscle cells by increasing the anti-apoptotic protein Bcl-2 and decreasing the pro-apoptotic protein, Bcl-associated X protein (BAX). Our previous study (Leung et al., 2002) also demonstrated the reduction of nitric oxide synthase (NOS) and the up-regulation of transforming growth factor beta 1 (TGF-β1) in brain tissue of LLI (660 nm, 8.8 mW) to rats with cerebral ischemia. The inhibition of NOS production (Gürsoy-Özdemir et al., 2000) and promotion of TGF-β1 (Gross et al., 1993) was found to reduce infarct lesion in cerebral ischemia. Here, we examined the protective mechanisms of LLI by studying the levels of various anti- and pro-apoptotic factors following cerebral ischemia. To the best of our knowledge, this is the first report demonstrating the effects of LLI on the expression of pro- and anti-apoptotic signals in cerebral ischemic injury.

6.3 Materials and methods

6.31 Induction of cerebral ischemia and reperfusion

Transient focal cerebral ischemia was induced by occluding the right middle cerebral artery (MCA) for 1 hour followed by reperfusion as previously described by Chen et al. (1986), with modifications (Leung et al., 2002). Briefly, male Sprague–Dawley rats, weighing 300g-350g, were anaesthetized with an intraperitoneal injection of ketamine (70 mg/kg,

Alfasan Nederland B.V.) and xylazine (7 mg/kg, Alfasan Nederland B.V.). A scalp incision was made at the midpoint between the right eye and ear, and the right temporalis muscle was dissected and retracted to expose the zygoma and squamosal bones. A 5 mm x 5 mm window was prepared with a constantly cooled surgical drill (Mototool, Dremel). The right MCA was occluded for 1 hour by a surgical clip and then released to allow reperfusion. The 1-hour middle cerebral artery occlusion (MCAO) was chosen because it is sufficient to induce cerebral infarct (Leung et al., 2002). The removed bone was replaced and the temporalis muscle and overlying skin were sutured. Throughout the surgical procedure, the rectal temperature was monitored and maintained at approximately 37 °C with the use of an overhead lamp. Fifteen minutes before and after MCAO, femoral arterial blood was sampled for pH, arterial partial oxygen (paO_2) and carbon dioxide ($paCO_2$) pressure measurements using the Blood Gas and Electrolyte System (ABL 505, Radiometer, USA). Following the procedure, the rats recovered at room temperature (21-23 ºC) with *ad libitum* food and water. All experimental procedures were approved by our institution's animal subjects ethical committee.

6.32 Laser irradiation

Immediately following MCAO, a 660-nm laser beam (average power 8.8 mW with a 1-min energy density of 2.64 J/cm^2 and pulse frequency of 10 kHz) (Omega Excel Laser, Omega Laser Systems LTD, U.K.) was directed through the burr hole onto the cerebrum from a distance of 5 mm. The laser beam was on a gallium aluminum arsenide probe with a divergence adaptor and delivered a spot area of 20 mm^2. The application site was similar to the traditional acupoint, ah-shi point, where the patient feels painful when acupuncture needle inserts. This protocol of laser light application was used because it suppresses the activity of nitric oxide synthase (NOS), up-regulates neuro-protective TGF-β1 (Leung et al.,

2002) and reduces infarct size (Yaakobi et al., 2001). The laser light exposure was 1, 5 or 10 min, delivering 2.64 J/cm^2 (n=8), 13.20 J/cm^2 (n=8) or 26.40 J/cm^2 (n=6), respectively. The stroke group (n=8) received the same surgical procedure but did not receive laser treatment, and the naive group (n=4) was not subjected to surgery or laser treatment. The animals were sacrificed by an overdose of ketamine and xylazine mixture on post-injury day 4 in order to measure Akt, pAkt, BAD, pBAD, and Bcl-2 as well as caspase 9 levels and caspase 3 specific activity. Four days post-injury was chosen to correspond with peak levels of NOS expression (Leung et al., 2002).

6.33 Quantification of Akt, pAkt, BAD, pBAD, Bcl-2 and Caspase 9 proteins by western blot analysis

The protein concentration of homogenate prepared from the right cerebral hemisphere was measured with the Bio-Rad Protein Assay (Bio-Rad Laboratories). In brief, 100 μg of soluble protein was resolved by SDS-polyacryamide gel electrophoresis (SDS-PAGE) and the separating gels were electro-blotted overnight at 4 ºC. The blots were incubated overnight at 4 ºC with rabbit antibodies against rat Akt, pAkt, Bad, pBad, Bcl-2 and caspase 9 (1:200 dilution, 0.5 μg/ml, Santa Cruz Biotechnology, Inc.). After reacting with Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.), chemiluminescence was captured by the UVP-Chemi System (UVP, Inc.), and signal intensities (expressed as ng/mg total protein) were analyzed using Lab-Works software (UVP, Inc.). Select blots were directly reprobed with anti–β-actin antibody (1:5000; Sigma Chemical Co.), and the resulting signal intensity was used to confirm equal sample loading and even transfer. The expression of apoptotic parameters is defined as ng/mg protein.

6.34 Functional assay of caspase 3 activity

Caspase 3 specifically cleaves at the C-terminal side of the aspartate residue in the target sequence DEVD (Asp-Glu-Val-Asp). The CaspACE Assay System Colorimetric Kit (Promega) was used to measure caspase 3 activity. Each 40-μl sample was incubated with 32 μl caspase assay buffer, 2 μl dimethyl sulfoxide, 10 μl 100 mM dithiothreitol and 2 μl colorimetric substrate (DEVD-pNA) for 4 hours at 37 °C. This substrate was labeled with chromophore p-nitroaniline (pNA). During the incubation reaction, pNA is released from the substrate by caspase 3, which serves as a DEVDase. The release of pNA results in the production of a yellow color, which was measured by absorbance at 405 nm with a micro-plate reader (BioTek Instruments, Inc.). The amount of free pNA was calculated according to a pNA standard with a range of 0-100 μM.

6.35 Statistical analysis

All data are presented as mean ± standard deviation (SD). The levels of various apoptotic parameters were compared using a two-way analysis of variance (ANOVA) for different treatment groups (5 levels). A probability value of less than 0.05 was considered significant for differences between groups of data, and significant ANOVA results were further analyzed using a post-hoc LSD test. SPSS version 14.0 (SPSS Inc.) was used for statistical analyses.

6.4 Results

6.41 Physiological parameters

The physiological parameters (paO_2, $paCO_2$ and pH) measured before and after transient

focal cerebral ischemia (Table 6.1) were comparable to results reported by others (e.g., Crack et al., 2003). Moreover, the blood gas values measured (paO_2, $paCO_2$ and pH) before and after ischemia were not significantly different, suggesting that the results were not due to hypoxia-induced acidosis (Ginsberg and Busto, 1989).

	paO_2 (mmHg)	$paCO_2$ (mmHg)	pH
Pre-ischemia (n= 6)	103.2 ± 5.8	45.6 ± 3.4	7.244 ± 0.024
Post-ischemia (n=6)	101.5 ± 13.8	44.9 ± 5.1	7.244 ± 0.035

Table 6.1 Arterial partial pressure of O_2 (paO_2) and CO_2 ($paCO_2$) and pH measured 15 min before (n=6) and after (n=6) ischemia.

6.42 The effect of laser treatment on Akt, pAkt, BAD, pBAD and Bcl-2

The level of Akt protein in the stroke group was 30.54 ± 1.45 ng/mg, which was not significantly different than the level of the naive group (22.09 ± 1.15 ng/ml) (Table 6.2). In contrast, following 1, 5 and 10 min LLI, Akt levels significantly increased ($P<0.001$) to 95.18 ± 17.08, 97.34 ± 36.25 and 113.05 ± 19.35 ng/mg, respectively (Table 6.2). Additionally, pAkt and Bcl-2 followed a similar trend after LLI (Table 6.2). The pAkt levels in the stroke and naive groups were 8.24 ± 0.66 and 5.59 ± 0.46 ng/mg, respectively. Following LLI for 1, 5 and 10 min, pAkt levels significantly increased ($P<0.001$) to 47.05 ± 16.53, 59.36 ± 3.66 and 62.13 ± 2.96 mg/ml, respectively. Lastly, Bcl-2 levels in the stroke

and naive groups were 0.93 ± 0.44 and 0.50 ± 0.06 ng/mg, respectively. Following LLI for 1, 5 and 10 min, Bcl-2 levels significantly increased (P<0.001) to 12.06 ± 4.07, 14.51 ± 5.25 and 12.82±6.20, respectively.

LLI did not have a significant effect on the expression of BAD (Table 6.2), as we recorded no significant difference in its expression among the naive, stroke and laser groups. However, LLI for 1, 5 and 10 min caused a significant increase in pBAD levels from 0.26 ± 0.03 ng/mg in stroke rats to 13.99 ± 6.59 (P<0.01), 21.81 ± 12.18 (P<0.001) and 11.98 ± 2.00 (P<0.01) ng/mg, respectively (Table 6.2).

Apoptotic factors	Naive (n=4)	Stroke (n=8)	1-min laser (n=8)	5-min laser (n=8)	10-min laser (n=6)
Akt (ng/mg protein)	22.09 ± 1.15	30.54 ± 1.45	95.18 ± 17.08**	97.34 ± 36.25**	113.05 ± 19.35**
p-Akt (ng/mg protein)	5.59 ± 0.46	8.24 ± 0.66	47.05 ± 16.53**	59.36 ± 3.66**	62.13 ± 2.96**
BAD (ng/mg protein)	21.46 ± 1.08	34.98 ± 3.07	40.74 ± 19.45	27.91 ± 16.32	35.00 ± 12.92
p-BAD (ng/mg protein)	0.25 ± 0.01	0.26 ± 0.03	13.99 ± 6.59*	21.81 ± 12.18**	11.98 ± 2.00*
Bcl-2 (ng/mg protein)	0.50 ± 0.06	0.93 ± 0.44	12.06 ± 4.07**	14.51 ± 5.25**	12.82 ± 6.20**

Table 6.2 Levels of various apoptotic markers measured from naive, stroke and stroke groups treated with 1, 5 or 10 min of low-energy laser irradiation.* represents P<0.01 vs. naive and stroke; ** represents P<0.001 vs. naive and stroke.

6.43 The effect of laser treatment on caspase 9 expression and caspase 3 activity

Four days after injury, the level of caspase 9 expression in the stroke group (7.98 ± 0.21 ng/mg) was significantly higher (P<0.001) than the naive group (2.56 ± 0.13 ng/mg). However, the level of caspase 9 expression decreased (P<0.001) after 1-min (3.55 ± 2.00 ng/mg) and 5-min (4.23 ± 1.17 ng/mg) LLI (Figure 6.1A, B).

Figure 6.1A

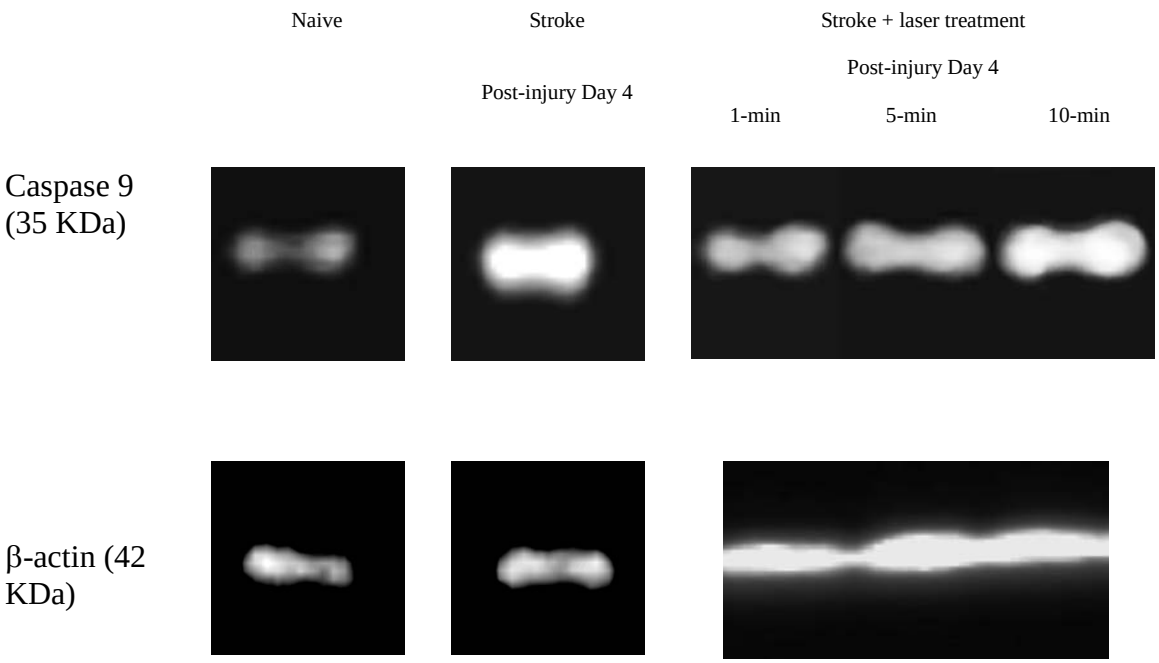

Figure 6.1B

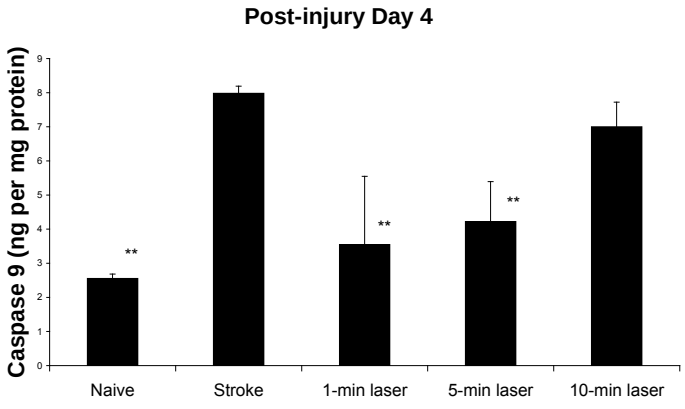

Figure 6.1 Expression of caspase 9. (Figure 6.1A) Western blot analysis of caspase 9 measured in naive rats and at post-injury Day 4 with or without 1-, 5- or 10-min low-energy laser irradiation. β-actin was used as an internal control. (Figure 6.1B) A summary of caspase 9 expression levels from the groups shown in figure 6.1A. ** represents P<0.001 vs. stroke from an ANOVA followed by LSD test.

Concomitantly, caspase 3 activity was also significantly increased by MCAO, with stroke and naive groups having an activity of 560.63 ± 24.56 and 165.15 ± 7.55 pmol pNA liberated/hour/mg protein, respectively (P<0.001). As with caspase 9 expression, caspase 3 activity decreased (P<0.001) with LLI after 1, 5 and 10 min to 214.02 ± 17.29, 224.67 ± 59.68 and 183.71 ± 5.89 pmol pNA liberated/hour/mg protein, respectively (Figure 6.2).

Figure 6.2

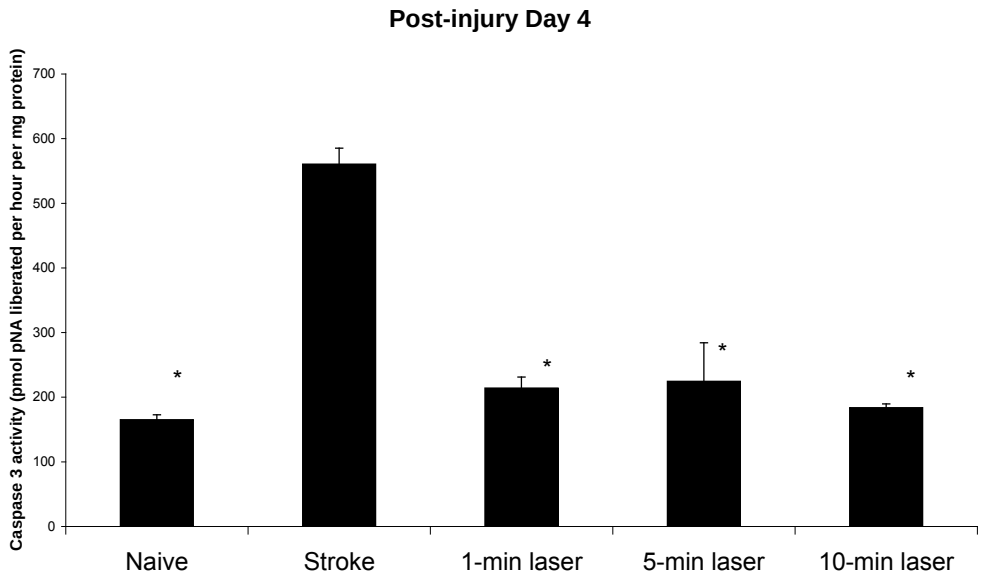

Figure 6.2 Caspase 3 activity measured by functional assay. Activity was measured in naive rats and at post-injury Day 4 with or without 1-, 5- or 10-min low-energy laser irradiation. ** represents P<0.001 vs. stroke from an ANOVA followed by LSD test.

6.5 Discussion

Here, we present a pilot study showing that low-energy laser irradiation (LLI) (1-min, 5-min and 10-min) reduced the expression or activity of pro-apoptotic factors and increased the expression of anti-apoptotic factors at 4 days post-injury in an animal model of cerebral ischemia and reperfusion. These biochemical changes observed are the consequences of the laser light treatment early after ischemia and could indirectly promote tissue protection by inhibition of apoptosis. Inhibition of apoptosis is believed to be effective in neuroprotection after cerebral ischemia. Linnik et al. (1993) demonstrated that the size of infarction produced by focal cerebral ischemia has significantly reduced by inhibition of programmed cell death through injection of protein synthesis inhibitor, cycloheximide. Neuroprotection also happens in the management of single apoptotic factor. Decreased infarct was observed in over-expressed Bcl-2 transgenic mice (Martinou et al., 1994), IL-1 beta converting enzyme (ICE) knockout mice (Schielke et al., 1998), BH3 interacting-domain death agonist (BID) knockout mice (Plesnila et al., 2001) and caspase inhibitors administration (Endres et al., 1998), whereas increased infarct was showed in Bcl-2 knockout mice (Hata et al., 1999).

Arterial blood gas analysis of rats 15 min before and after transient focal cerebral ischemia revealed no significant effect of ischemia-reperfusion on paO_2, $paCO_2$ and pH, suggesting that the experimental procedure used here did not cause acidosis and that the observed changes in the cerebral tissues were likely due to the ischemia-reperfusion challenge itself. This is consistent with previous studies (Ginsberg and Busto, 1989).

Increased intracellular calcium triggers detrimental cascades, such as nitric oxide

formation and apoptosis (MacManus and Linnik, 1997). Calcium can trigger the release of mitochondrial proteins, such as cytochrome c, apoptosis protease-activating factor-1 and other apoptosis-inducing factors. In turn, these factors subsequently activate initiator caspases (e.g., caspase 9) and effector caspases (e.g., caspase 3) (Green and Reed, 1998), culminating in DNA fragmentation resulting from cerebral ischemia (Linnik et al., 1995; Yao et al., 2001). Neuron survival can be promoted by up-regulating anti-apoptotic factors, such as Akt and pAkt, which inhibit apoptotic signals, such as caspase 9 (Wang et al., 2002) and BAD (Datta et al., 1999). A recent study showed that reducing phosphatidyl inositol 3 kinase/Akt expression both enhanced caspase 3 activation and aggravated removal injury after cerebral ischemia (Weise et al., 2006). In addition, Bcl-2 prevents the expression of apoptosis-specific proteins, thus serving to protect cells from apoptosis (Grand et al., 1995).

As our results show, LLI up-regulated Akt, pAkt and Bcl-2, thus effectively minimizing apoptotic cell death. The LLI was applied immediately after MCAO, which may be important because early activation of Akt during the first 24 hours may delay ischemia-induced neuronal death (Ouyang et al., 1999). A therapeutic window within the first hour following MCAO has been suggested for attenuating neuronal damage (Schulz et al., 1999). The use of LLI has been reported to suppress NOS activity (Leung et al., 2002). NOS activity causes apoptosis (Dalkara et al., 2000) through peroxynitrite formation (Gürsoy-Özdemir et al., 2000), which up-regulates the expression of TGF-β1. The expression of TGF-β1 promotes neuronal survival and neurite growth, regulates Schwann cells and inhibits astroglial cell division after a stroke (Unsicker et al., 1992). The anti-apoptotic effects of TGF-β1 can be explained by an up-regulation of Bcl-2 expression (Prehn et al., 1994; Unsicker et al., 1992).

Our results indicate that 1–5 -min of LLI can restore caspase 9 expression and caspase 3 activity to non-injured levels four days post-injury. Thus, the neuro-protective effect of LLI appears to be dosage independent. Similar studies have shown that laser treatment has no dosage dependence for reducing and enhancing NOS and TGF-β1 expression, respectively (Leung et al., 2002). Nevertheless, the paramount importance of caspase inhibition in protecting the brain from cerebral ischemia damage that accompanies neurodegenerative disorders (Schulz et al., 1999) must be emphasized. This inhibition may relate to the potential role of the laser in suppressing inducible NOS (Leung et al., 2002), which catalyzes the production of nitric oxide (NO), an oxidative free radical for inflammation. In our study we deliberately used a divergence laser adaptor, allowing us to spread the beam over a 20 mm^2 area, thereby avoiding cell death via thermal energy (Bloom et al., 2001; Tirlapur et al., 2001; Varriale et al., 2000). Other possible neuro-protective mechanisms provided by LLI include the up-regulation of c-Fos expression (Greco et al., 2001), the restoration of compound action potentials (Rochkind et al., 1991) and the prevention of degenerative changes in spinal cord neurons by astrocyte and oligodendrocyte proliferation (Rochkind et al., 1990).

6.6 Conclusion

In conclusion, these data provide compelling evidence to support the idea that treatment with low-energy lasers can play an important therapeutic role by up-regulating Akt, pAkt, pBAD and Bcl-2 expression and down-regulating caspase 9 expression and caspase 3 activity. These results provide a rationale for using therapeutic laser irradiation in human patients following an ischemic event, such as stroke. Further investigation is needed to optimize the timing and other parameters of the laser treatment following cerebral ischemia; nevertheless, the application of low-dose laser irradiation may be an important treatment in conjunction with other neuro-protective agents for the treatment of stroke.

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Chapter 7

Discussion

7.1 The mechanism of acupoint stimulation

The current study demonstrated the effectiveness of various forms of acupoint stimulation on cerebral ischemia. However the mechanism of action of acupuncture is still unclear and what is being stimulated at the acupoint still remains question.

Kawakita (1993) suggested a hypothesis of polymodal receptor (PMR) to explain the mechanism of acupuncture. PMR is a type of nociceptor and responsive to mechanical, thermal and chemical stimuli. The original concept of PMR came from Kumazawa and Mizumura (1977) who investigated the responses to mechanical stimulation, intra-arterial injection and local application of chemical solutions, and thermal stimulation of the surface of the muscle in dog. Receptors responded to all types of stimuli were observed in the thin fibre afferents of the muscle. The responsive region to mechanical stimulation was spot-like and appeared to be located on the surface of skin and in the midst of muscle. Each responsive region contained different threshold and the pattern of discharge of the mechanical response. Heating the receptive area of the muscle surface caused responses in some units which were sensitive both to mechanical and to chemical stimulations. The threshold varied from 38.0 °C to 48.3 °C. The mean values are 43.1 °C for C fibre units and 41 °C for A-delta fibre units.

Needle puncture of classical manual acupuncture stimulates mechanoreceptor. Infarct volume was depleted in middle cerebral artery occluded rat after acupuncture intervention

(Zhai et al., 1993).

Thermal stimulus such as moxibustion is another complementary alternative medicine choice. Moxibustion treats patient with heat generated by burning herbal preparations containing Artemisia vulgaris to stimulate acupuncture points. Moxibustion was found to be effective in stroke rehabilitation. Chen et al. (2006) treated patients with moxibustion at acupoints ST 36 and GB 39 for 20 days. When compared to control group who was given routine treatment, moxibustion at Zusanli (ST 36) and Xuanzhong (GB 39) demonstrated significant improvement in cerebral blood flow and functional status.

A number of previous studies also investigated the effect of chemical stimulation on acupoint. Chiang et al. (1973) injected anesthetic drugs into a muscle nerve bundle innervating the acupoint LI 4 completely exterminated the de-qi sensation, but the sensation was not abolished by anesthetizing a cutaneous nerve. Kong et al. (2010) examined the effect of acupoint-injection of Chuanxiongzine on the expression of urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) in the cerebral cortex in rats with cerebral ischemia-reperfusion injury. uPA is used clinically as a thrombolytic agent as tissue plasminogen activator (tPA) which catalyzes plasminogen to plasmin. Injection of Chunanxiongzine solution to acupoint GV 20 and GV 14 was found to up-regulated the production of cortical uPA and down-regulated the production of its inhibitor, PAI-1. Chen et al. (2011) studied the effect of acupoint-injection on the expression of Bcl-2 and Bax in cerebral ischemia rat. Angelica-root solution was injected to acupoint GV 20 and GV 14 after modeling. The number of Bcl-2 and Bax-positive cells of treatment group was significantly increased in ischemic area when compared to the control group.

Some scientists demonstrated the uniqueness of acupoint by histological means. Abraham et al. (2011) examined the distribution of transient receptor potential vanilloid type-1 (TRPV1) receptor in the skin regions of acupoint (BL 40) compared to the non-point (along the meridians) and the non-meridian control (adjacent to but not along the meridians) under electroacupuncture. They found that in the non-stimulated situation, the number of TRPV-1-positive nerve fibres in acupoint was significantly higher than the non-meridian control region. Only in the acupoint region, stimulation could significantly up-regulate the outcome measure. The result suggested that TRPV1 may take part in mediating the effect of EA through the sensory afferent to the central nervous system.

Advanced imaging technology also widened our horizon of the effect of acupuncture on brain activity. Kong et al. (2002) used functional magnetic resonance imaging (fMRI) to investigate the brain regions involved in electroacupuncture and acupuncture stimulation. Acupuncture needle manipulation and electroacupuncture stimulation were performed at acupoint LI 4 to corresponding groups. Results showed that acupuncture mainly produced fMRI decreasing signal in posterior cingulate, superior temporal gyrus and putamen/insula. In contrast, electroacupuncture mainly produced fMRI increasing in precentral gyrus, postcentral gyrus/inferior parietal lobule, and putamen/insula. These results indicated that different brain regions were involved in manual acupuncture and electroacupuncture. Stimulation of acupoint ST 36 by manual acupuncture and electroacupuncture also produced more fMRI responses than placebo-like tactile control stimulation did (Napadow et al., 2005).

7.2 Comparison of different forms of acupoint stimulation

Although the current study investigated various forms of acupoint stimulation on the effect of neuroprotection, no comparison between these therapies was done. Previous studies showed that more favourable effect was observed on electroacupuncture than manual acupuncture. Tsui and Leung (2002) compared the effectiveness of manual acupuncture (MA) and electro-acupuncture (EA) on 20 patients with chronic tennis elbow. A single-blinded randomized controlled trial was performed on 20 patients. The acupuncture points of GB 34 and ST 38 were used in both treatment groups. After 6 sessions of treatment, significant difference observed in pain relief illustrated electro-acupuncture may be superior to manual acupuncture in treating patients with tennis elbow. Hwang et al. (2010) compared the effect of neurogenesis of acupuncture and electroacupuncture (EA) in normal rat. Both intervention were applied in the acu-points, ST 36 and GV 20, once a day for 3 weeks. When compared to the control and sham acupuncture groups, both treatment groups demonstrated significant increase in the number of Ki67-positive cells (marker of cell proliferation) and doublecortin (DCX)-positive cells (marker of neurogenesis). EA was found to have greater effects on neuroblast plasticity than acupuncture.

Acupuncture or electroacupuncture seems to be favorable in treatment effect than other forms of stimulation. Ng et al. (2003) compared the therapeutic effect of electroacupuncture and transcutaneous electrical nerve stimulation (TENS) on 24 elderly with osteoarthritic (OA)-induced knee pain. After 8 sessions of treatment, significant reduction of knee pain was observed in both EA group and TENS group. However, significant improvement of the functional mobility and balance, measured by timed Up-and-Go Test (TUGT) score, was only observed in EA group. Choy et al. (2010) studied the

differences in brain responses between pressure and acupuncture stimulation at the acupoint LI 11 and ST 36 using functional magnetic resonance imaging (fMRI). In acupoint LI 11, parahippocampal gyrus, cerebellum, left side of thalamus, and right side of posterior cingulate regions were more activated by acupuncture stimulation than pressure. Similar result was obtained in ST 36 stimulated fMRI signals at the secondary motor cortex, limbic system (cingulate gyrus, posterior cingulate), primary visual cortex, pons, and medulla regions.

7.3 Choice of acupoint

The current results illustrated that appropriate selection of acupoint is important in the efficacy of treatment. In chapter 4, we compared the effectiveness of electroacupuncture (EA) at ST 36, GB 20 and non-acupoint. Only ST 36-stimulated group demonstrated significant up-regulation of both Bcl-2 and TGFβ-1. Similar finding was observed in chapter 5. Pre-ischemia high voltage electrical stimulation (HVES) was performed at ST 36, LI 10 and ST 36 plus LI 10. Neuroprotection through down-regulation of iNOS and MDA were only observed in ST36-stimulated subjects. This acupoint-specific characteristic was in concert with previous pre-ischemia study of EA which also indicated that the selection of non-acupoint was not only non-beneficial but harmful in terms of the production of MDA (Siu et al., 2004). Qian et al. (2009) also studied the specificity of acupoints in the acupuncture treatment of ischemic cerebral infarction. Cerebral blood flow (CBF) was the outcome measure to compare the effectiveness of specific acupoint. After acupuncture treatment, CBF was significantly increased in the GV 20 group and the PC 6 groups, but did not significantly increased in the LU 5 group, SP 6 group and BL 40 groups. Therefore, without extensive study in individual acupoint on different disease,

judgment of application of acupoint stimulation such as acupuncture, electroacupuncture or TENS of specific disease may be debatable.

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Chapter 8

Conclusion

The current study demonstrated that acupoint stimulation in various types may be effective in cerebral ischemia neuroprotection. However, the protective effect may be acupoint specific. Selection of acupoint is important in the efficacy of treatment. Scientific research is needed in further to clarify individual and combined application of acupuncture point.