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EFFECTS OF ELECTROACUPUNCTURE ON THE OXIDATIVE STRESS IN ADULT RATS AFTER CEREBRAL ISCHEMIA

SIU KA WAI FLORA

Ph.D.

THE HONG KONG POLYTECHNIC UNIVERSITY

2005
THE HONG KONG POLYTECHNIC UNIVERSITY

DEPARTMENT OF REHABILITATION SCIENCES

EFFECTS OF ELECTROACUPUNCTURE ON THE OXIDATIVE STRESS IN ADULT RATS AFTER CEREBRAL ISCHEMIA

SIU KA WAI FLORA

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

FEBRUARY 2004
Cerebrovascular disease, especially stroke, is the third leading cause of death in Hong Kong. The conventional treatment and rehabilitation cannot effectively shorten the time for recovery of stroke patients. Another possible treatment, electro-acupuncture (EA), has been used for treating patients with stroke in China. The lack of scientific basis on evaluating its effectiveness restricts its use on patients with stroke in Western countries. This study, therefore, is aimed at investigating its effectiveness on regulating the reactive oxygen species-mediated injury and the apoptotic neuronal damage induced by ischemia-reperfusion in adult rats. Firstly the animal model was induced by the occlusion of middle cerebral artery (MCAO) for 1 hour followed by reperfusion for 1 to 5 days. The severity of model was assessed by the activity and expression of nitric oxide synthase (NOS), activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx), and the resulting lipid peroxidation (LPX) levels. Results showed that the activity and expression of NOS was increased significantly at day 4 while the activities of SOD and GPx were suppressed, leading to the exacerbation of LPX, which confirmed the successful induction of MCAO in rats. In the second experiment, to investigate the regulation on reactive oxygen species-mediated neurotoxicity, EA stimulation (30 min, 2 Hz, 0.7V) was immediately applied after MCAO. Two pairs of acupuncture points, Fengchi (GB20) and Zusanli (ST36), were individually used for comparison. In comparison to the ischemic...
condition, inhibited activity and expression of NOS were observed at day 4 after EA stimulation \((P<0.05)\). The concomitant increase of activities of SOD and GPx started as early as 1 day after MCAO and maintained at day 4 \((P<0.05)\). The upregulation of manganese SOD expression was likely due to the increased thioredoxin expression as observed in the following experiment \((P<0.05)\). Owing to the higher removal of reactive oxygen species and diminished formation of peroxynitrite, the LPX levels were greatly attenuated \((P<0.05)\). In the third experiment, the protective effect of EA stimulation against protein oxidation induced by ischemia-reperfusion was monitored by the activity of thioredoxin reductase (TR) and the expression of thioredoxin (Trx). Results showed that EA stimulation could only slightly stimulate the TR activity, but greatly increase the Trx expression \((P<0.05)\), suggesting that the EA stimulation was able to prevent protein oxidation by potentiating the functional level of Trx/TR system. The anti-apoptotic effect of EA stimulation was also investigated in the fourth experiment. Results found that ischemic rats with EA stimulation expressed more anti-apoptotic proteins (TGF\(\beta\)-1, Bcl-2 and pAkt) but less apoptotic proteins (caspase-3 and -9). This down-regulation was likely due to the inhibited BAX translocation and the subsequent release of cytochrome c. Furthermore, the effect of the time to deliver EA stimulation was investigated by the delayed post-ischemia EA stimulation which was applied 1 to 3 days from the onset of MCAO. There was no observable difference between rats with immediate EA stimulation and delayed EA stimulation, suggesting that the effect of EA stimulation was not highly governed by the time. With regard to the clinical consideration, the effect of pre-ischemic EA stimulation was also investigated by giving 3- or 18-times EA stimulation prior to MCAO. The neuroprotective effect was still observed. To sum up, the EA stimulation at GB20 or ST36 could ameliorate the ischemia-reperfusion injury, in which EA stimulation at ST36 produced a better result.
CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces 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.

SIU KA WAI FLORA
PUBLICATIONS ARISING FROM THE THESIS

**Full-Length Referred Publications In Scientific Journals:**


4. **Siu FKW**, Lo SCL, Leung MCP, 2005. Effects of pre-ischemic electroacupuncture on regulating nitric oxide synthases and transforming growth factor beta-1 in adult rats. *(Submitted)*


6. **Siu FKW**, Lo SCL, Leung MCP, 2005. Effect of EA stimulation on regulating the apoptosis in induced by cerebral ischemia. *(In preparation)*

**Associated Full-Length Referred Publications In Scientific Journals:**


**Conference Papers To Scientific Meetings:**


**Award Received From International Scientific Meeting:**

1. The International Society for Neurochemistry (ISN) Travel Award issued by 3rd Asia Pacific Symposium on Neural Regeneration, December 3-5 2002, Perth, Australia.

**Scholarship Awarded by The Hong Kong Polytechnic University:**

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<td>Akt, pAkt</td>
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<td>BAX</td>
<td>Bcl-associated X protein</td>
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<td>Bcl-2</td>
<td>B-cell lymphoma/leukaemia-2</td>
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<td>CaCl₂</td>
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<td>EA</td>
<td>Electroacupuncture</td>
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<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
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<td>FAD</td>
<td>Flavin adenosine dinucleotide</td>
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<td>GSH, GSSG</td>
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<td>IgG</td>
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<td>KDa</td>
<td>Kilo-dalton</td>
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<td>MCA</td>
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<td>Occlusion of middle cerebral artery</td>
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<td>MDA</td>
<td>Malondialdehyde</td>
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<td>NO</td>
<td>Nitric oxide</td>
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<td>NOS</td>
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<td>nNOS, iNOS, eNOS</td>
<td>Neuronal, inducible, endothelial nitric oxide synthase</td>
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CHAPTER 1

Introduction

1.1 STROKE

1.1.1 General consideration

Stroke is the second leading cause of death in the world. It is a disease that predominantly occurs in the adult and the elderly. According to the statistics reported by World Health Organization (2003), it was estimated that the cerebrovascular disease was accounted for 5.5 million deaths worldwide which was equivalent to 9.6% of all the deaths. Two-thirds of these fatal subjects occurred in the people living in developing countries and 40% of the subjects were aged less than 70 years. Similarly, stroke is the third leading cause of death in the United States of America [American Heart Association, 2001]. There are about 600,000 Americans suffering from a new or recurrent stroke (cerebrovascular accident) in each year. New cases of patients suffering from stroke occur in every 53 seconds and the death of those patients appears in every 3.1 minutes. In China and Hong Kong, the cerebrovascular disease (stroke) has been ranked as the top three leading cause of death for more than 20 years [Government of The People’s Republic of China, 2000; Hong Kong Government, 2000]. Annually the number of deaths contributes to over 16% of total death. The high rate of stroke incidence increases the costs for medical treatment and health care [Ebrahim and Harwood, 1999]. Therefore, a considerable number of research studies are established in understanding stroke-induced brain damage and in developing the treatment strategies for patients with stroke.
1.1.2 Classification of stroke

According to the recommended standard by World Health Organization (2003), stroke is defined as “a focal (or at times global) neurological impairment of sudden onset, and lasting more than 24 hours (or leading to death) and of presumed vascular origin”. This definition excludes the transient ischemic attack (TIA), which is defined as focal neurologic symptoms lasting less than 24 hours. In addition, surdural hemorrhage, epidural hemorrhage, poisoning and symptoms caused by trauma are also excluded. There are three major types of stroke: Ischemic stroke, intracerebral hemorrhage and subarachnoid hemorrhage. Each type can produce clinical symptoms that fulfill the definition of stroke.

1. Ischemic stroke is caused by a sudden occlusion of arteries supplying the brain [World Health Organization, 2003]. The occlusion may either be due to a thrombus formed directly at the site of occlusion (thrombotic ischemic stroke), or a thrombus formed in another part of the circulation which follows the bloodstream until it obstructs arteries in the brain (embolic ischemic stroke). The diagnosis of ischemic stroke is usually based on neuro-imaging recordings, but it may not be possible to decide clinically or radiologically whether it is a thrombotic or embolic ischemic stroke [Ebrahim and Harwood, 1999].

2. Intracerebral hemorrhage is a bleeding from one of the cerebral arteries in the brain [World Health Organization, 2003]. The lesion causes symptoms that mimic those seen for ischemic stroke. Its diagnosis depends on access to neuroimagining where it can be differentiated from ischemic stroke [Ebrahim and Harwood, 1999].

3. Subarachnoid hemorrhage is characterized by arterial bleeding in the space between the two meninges, pia mater and arachnoidea [World Health Organization, 2003].
Organization, 2003]. Typical symptoms are the sudden onset of very severe headache and usually impaired consciousness. The diagnosis can be established either by neuro-imaging or lumbar puncture [Ebrahim and Harwood, 1999].

1.1.3 Causes of stroke
Stroke is a multi-factorial disease where many risk factors have been described. These risk factors can be generally classified into modifiable and non-modifiable. The typical examples of non-modifiable risk factors are age and sex. Usually, high age and male sex increase the risk level [World Health Organization, 2003]. On the other hand, the modifiable factors are those individual factors that can be changed, including tobacco smoking, sedentary lifestyle, diet, alcohol, high blood pressure, and factors in environment such as passive smoking, places of residence, climate, and air quality [Ebrahim and Harwood, 1999; World Health Organization, 2003]. In addition, the risk of stroke could be increased greatly when two or more risk factors are combined. For example, smoking increases the risk of stroke by two to three times, and hypertension increases the risk by about five times. The combined effects of both smoking and hypertension might be estimated to cause between eight- and fifteen-fold of increased risk [Ebrahim and Harwood, 1999]. The risk of stroke can be highly increased if individuals have previous transient ischemic attacks, ischemic heart disease and atrial fibrillation [Ebrahim and Harwood, 1999].

1.1.4 Conventional treatment of stroke patients
When the patients with stroke are sent to the hospital, the process for diagnosing early stroke and providing intervention is then employed [Cohen, 2000]. The process includes three components, diagnosis, management and prognosis. Lists of
information (Table 1.1) are recommended to collect for use in its diagnosis, management and prognosis [Ebrahim and Harwood, 1999]. The use of the information would help designing which treatment should be used. In the diagnostic stage, the evaluation is based on the physical examination and the neuro-imaging obtained from the magnetic resonance imaging (MRI) [Cohen, 2000; Miller, 1999]. The initial physical examinations are to determine the patient level of consciousness, stroke severity, stroke localization and stroke etiology [Cohen, 2000]. The use of MRI is to determine the localization of tissue abnormalities after stroke attack [Cohen, 2000; Ebrahim and Harwood, 1999]. The management of patients with stroke is usually determined by the time from the onset of stroke. For the patients suffering from stroke for the first six hours, the management including the control of respiration, blood pressure, and restricted supply of glucose and fluid is recommended [Cohen, 2000; Ebrahim and Harwood, 1999]. Respiratory management is aimed to increase the systemic oxygen desaturation which exacerbates the brain injury originating from focal cerebral hypoxia. Blood pressure control is useful to decrease the elevated blood pressure during an episode of cerebral ischemia as chronic hypertension is an independent risk factor for the occurrence of stroke. Management of fluid and glucose supply is aimed to alleviate the cerebral edema and hyperglycemia [Cohen, 2000; Ebrahim and Harwood, 1999]. In addition, a combined therapy to prevent the formation of thrombus includes the administration of thrombolytic agents, anti-platelet agents and anti-coagulants. The commonly used thrombolytic agent is the tissue plasminogen activator (TPA) [Cohen, 2000; Miller, 1999]. It is a serine protease endogenously released in human tissue from vascular endothelium and preferentially activates plasminogen bound to fibrin, so as to minimize the systemic fibrinolytic activation and the formation of thrombus [Cohen, 2000; Ebrahim and
The use of anti-platelet agents and anticoagulation agent is to reduce the aggregation of platelets and fibrin that initiates the recurrent embolization. Subsequently, for the patients suffering from stroke for six hours to three days, a further management is used to avoid the formation of intraluminal arterial thrombus and arterial stenosis, which can be identified by arteriography [Cohen, 2000; Ebrahim and Harwood, 1999]. Other management strategies to prevent the cerebral edema, seizure, blood pressure fluctuation, and hyperthermia are also applied if necessary. However, the patients with stroke survived from stroke can still be suffering from disabilities or even handicaps [Ebrahim and Harwood, 1999]. The disabilities can be improved by a series of rehabilitation processes to increase the motor function, speech ability, and memory [Cohen, 2000]. In here, “rehabilitation” is defined as the combined and coordinated use of medical, social, educational, and vocational measures to train or retrain individuals to the highest possible level of functional ability [World Health Organization, 2003]. The purpose of rehabilitation is to reduce the burden of illness on the patients, his family and the society [Cohen, 2000; Ebrahim and Harwood, 1999; Miller, 1999]

1.1.5 Implication of experimental stroke model

Although the current conventional management of stroke can protect patients from death, it is not possible to eliminate the associated disability. It takes long-term rehabilitation to achieve a significant improvement, which also creates a huge burden to both patients and the health care sector [Cohen, 2000; Ebrahim and Harwood, 1999; Miller, 1999]. In this regard, the cellular and molecular basis of stroke is essential to find out possible ways to reduce the injury of cerebral ischemia, and further to evaluate their effectiveness on improving the outcomes of cerebral ischemia.
Table 1.1. A classification of information for use in its diagnosis, management, and prognosis of stroke [Adopted from Ebrahim and Harwood, 1999]

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Management</th>
<th>Prognosis</th>
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<tbody>
<tr>
<td>Side of weakness</td>
<td>Side of weakness</td>
<td>Speech impairment</td>
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<td>Speech impairment</td>
<td>Speech impairment</td>
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<td>Swallowing</td>
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<td>Mode of onset</td>
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<td>Headache</td>
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<td>Vomiting</td>
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<td>Photophobia</td>
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<td>Pre-stroke ability</td>
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<td>Type of stroke</td>
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1.1.6 Animal models for stroke

In humans, stroke is characterized by a sudden blockade of blood flow into the brain [Hou and MacManus, 2002]. There are basically two types of stroke: global ischemia and focal ischemia. Global ischemia is usually resulted from the cardiac arrest or other causes of collapse of the systemic circulation [Cohen, 2000]. Focal ischemia is the focal loss of blood flow to the brain due to the blockade of blood vessels [Cohen, 2000]. Accordingly, animal models have been developed to mimic the human cerebral ischemia. Relevant to the human cardiac arrest, the model of global ischemia involves the bilateral occlusion of the carotid and vertebral arteries for a short duration [Miller, 1999]. The model of focal ischemia involves the occlusion of the middle cerebral artery (MCA) either permanently or transiently that was in close resemblance to the human cerebral ischemia [Miller, 1999].

Occlusion of middle cerebral artery (MCA) can then be generally classified into four types referring to the site of MCA occlusion [Fox, 1993; Gibo, 1981; Skoczylas and Wiland, 1999; Stritch School of Medicine, 2003] (Figure 1.1):

1. Occlusion at the Horizontal M1 segment leads to the contralateral hemiplegia of motor function (where face, arm and lower extremity are less affected) and homonymous hemianopia of sensory function.

2. Lateral Lenticulostriate A is the branch of M1 segment of MCA that supplies the basal ganglia, namely caudate, globus pallidus, putamen, and the posterior limb of the internal capsule. Its occlusion leads to damage of the internal capsule resulting in contralateral hemiparesis and sensory deficits. Speech as well as visual function may be affected.
Figure 1.1  Classification of occlusion of middle cerebral artery: (A) Horizontal M1 segment; (B) Lateral Lenticulostriate Arteries; (C) Sylvian M2 segment; and (D) Cortical M3 segment. [Adopted from Stritch School of Medicine, 2003]
3. Sylvian M2 segment, which divides into superior and inferior divisions, supplies nutrients and oxygen to the temporal lobe and insular cortex, parietal lobe and inferolateral frontal lobe. Occlusion of its superior division leads to brachiofacial paralysis which is a sensorimotor deficit involving the face and arm, (leg and foot to a lesser extent), motor aphasia and no impairment of alertness. Occlusion of its inferior division leads to superior quadrantanopia or homonymous hemianopia, aphasia and visual neglect.

4. Cortical M3 segment is the distal branches of MCA lateral to the insular cortex and loops around the operculum. Occlusion or embolization of individual cortical branches can produce highly circumscribed infarctions accompanied by specific neurologic deficits.

### 1.1.7 Mechanism of neuronal death following stroke

Cell death is generally described as apoptosis and necrosis based on a set of morphological criteria. Apoptosis is characterized by typical morphological changes including membrane blebbing, chromatin condensation into punctuate globules, cell shrinkage and disassembly into membrane-enclosed vesicles, apoptotic bodies [Kerr et al., 1972]. In contrast to apoptosis, necrosis produces the changed nuclear morphology and ruptured cellular membrane, and is characterized by the perturbation of mitochondrial function, the activation of proteases and endonucleases, and the alteration in gene expression [Nicotera and Leist, 1997]. Usually necrosis is found in ischemic core, while apoptosis is identified in penumbral core. Based on the appearance of distinct terminal morphologies, it is considered that necrosis and apoptosis are derived from two distinct biochemical pathways. However, such a
belief has been challenged by demonstrations of mixed morphological and biochemical characteristics of apoptosis and necrosis. In relation to the ischemia-reperfusion injury, the mechanism of cell death is now generally identified as three pathways that involve the excitotoxicity of glutamate, the implication of free radicals and the activation of apoptotic genes. As detailed below, these three pathways are highly related with each other.

1.1.7.1 Glutamate-induced neuronal death

Glutamate is the predominant excitatory neurotransmitter in the central nervous system. Under normal physiological conditions, the extracellular concentrations of glutamate are properly maintained [Bouvier et al., 1992]. Receptors for glutamate are classified as metabotropic and ionotropic. Ionotropic glutamate receptors are further categorized into N-methyl-D-aspartate (NMDA) receptor and non-NMDA receptors. During ischemia, the inadequate supply of glucose to neurons leads to a reduction in ATP levels causing depolarization of the pre-synaptic membrane and thereby increasing the release of glutamate [Sattler and Tymianski, 2001; Zipfel et al., 2000]. The extracellular glutamate level increases substantially and over-stimulates the receptors. It then promotes the Na\(^+\) influx and K\(^+\) efflux through the glutamate receptor-activated membrane channels. NMDA receptor-gated ion channels are highly permeable to Ca\(^{2+}\) ions and mediate Ca\(^{2+}\) influx into neurons. These lead to an increased calcium load, membrane depolarization, and neuronal cell body swelling. Alternatively, the non-NMDA receptor gates do not change Na\(^+\) and K\(^+\) conductance, but induce Ca\(^{2+}\) influx indirectly by alleviating voltage-dependent Mg\(^{2+}\) block of the NMDA receptor [Lee et al., 2000].
1.1.7.2 Free radicals-mediated neurotoxicity

A free radical is simply a molecule containing an odd number of electrons and thus may be considered to contain an open bond or a half bond, rendering it chemically reactive [McCord, 1985]. Intracellular free radicals are often considered as reactive oxygen species (ROS) which are chemically reactive molecules derived from oxygen [Betteridge, 2000; Fridovich, 1999; Halliwell, 1996, 1999]. There are at least five ROS molecules identified:

1. Superoxide anion ($O_2•^-$) created from molecular oxygen by the addition of an electron is not highly reactive. In ischemia-reperfusion, superoxide anion ($O_2•^-$) is produced endogenously by the activated flavoenzymes (xanthine oxidase) [Kuppusamy and Zweier, 1989], lipoxygenase and cyclooxygenase [Kuppusamy and Zweier, 1989; Zimmermann and Granger, 1994]. Two molecules of superoxide anion rapidly dismutate to form hydrogen peroxide and molecular oxygen.

2. Hydrogen peroxide ($H_2O_2$) has an ability to penetrate biological membranes. It plays radical-forming role as an intermediate in the production of more reactive ROS molecule, hydroxyl radical ($•OH$), by the action of myeloperoxidase. It is an enzyme present in phagosomes of neutrophils [Winterbourn et al., 2000].

3. Hydroxyl radical ($•OH$) creates a more damaged biological system than other ROS because of its strong reactivity with biomolecules. The radical is formed from hydrogen peroxide in the reaction catalyzed by metal ions released from ferritin ($Fe^{2+}$ or $Cu^+$) according to the Fenton reaction as below [Harris et al., 1994]:

$$H_2O_2 + Cu^+/Fe^{2+} \rightarrow •OH + OH^- + Cu^{2+}/Fe^{3+}$$
4. Nitric oxide (NO) is formed when nitric oxide synthase (NOS) is activated by intracellular calcium. There are three NOS isoforms that are named after the tissue from which they were first cloned [Bredt and Snyder, 1994; Marletta, 1994]: Neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). nNOS and eNOS are constitutively expressed and are calcium-dependent. Inducible NOS is expressed after immunologic challenge or neuronal injury and is calcium-independent. NOS catalyzes the stoichiometric conversion of L-arginine to NO and citrulline in the presence of oxygen and NADPH.

5. Peroxynitrite (OONO$^-$), which is highly cytotoxic, is formed by the reaction of superoxide anion and NO [Valdez et al., 2000]. Peroxynitrite can react directly with diverse biomolecules in one- or two-electron reactions, readily react with CO$_2$ to form highly reactive nitroso-peroxocarboxylate or protonated as peroxonitrous acid and undergo homolysis to form •OH and •NO$_2$.

These ROS can readily react with most biomolecules, such as DNA, lipids and proteins, by initiating a chain reaction of free radical formation. Firstly, in the chemical modification of DNA, ROS, especially •OH, induces the mutagenesis [Nordberg and Arner, 2001]. Secondly, in reacting with the lipid, the process of lipid peroxidation is initiated. Having the multiple double bonds, polyunsaturated fatty acids are excellent targets for free radical attack [Nordberg and Arner, 2001]. Thirdly, the ROS can react with those sulphur- or selenium-containing residues, resulting in the modification of protein structures.
1.1.7.3 Apoptotic injury

Free radicals generated trigger the activation of the apoptotic injury. The initiation of death receptor proteins on cell membrane and the subsequent involvement of caspase (aspartate-specific cysteine proteases) cascade lead to apoptosis. A schematic diagram illustrating the general pathway of apoptosis induced by cerebral ischemia is shown in Figure 1.2. Briefly the generation of signal for cell death, in the form of cytokine, is indirectly mediated by free radicals [Bonfoco et al., 1995; DeGracia et al., 2002; Du et al., 1996; Eldadah and Faden, 2000; Graham and Chen, 2001; Hou and MacManus, 2002; Kirino, 2000; Mattson et al., 2000]. The binding of cytokines (as indicated as Step 1) to the membrane receptor generates the signal for death process (Step 2). It starts from the activation of the Bcl-2-associated death protein (BAD), which is normally present in the phosphorylated form (Step 3) [Rickman et al., 1999] (Stage 1 as indicated in Figure 1.2). The dephosphorylated BAD protein subsequently dimerizes with the antiapoptotic Bcl-XL protein that is constitutively bound to BAX (Bcl-associated X protein) [Henshall et al., 2002]. During this binding, the displaced BAX protein translocates to the mitochondrion and stimulates the release of cytochrome c [Graham and Chen, 2001] (Step 4). The released cytochrome c promotes the cleavage of pro-caspase-9 into activated caspase-9 that activates the activity of caspase-3 [Eldadah et al., 2000; Graham and Chen, 2001; Harrison et al., 2001; Mouw et al., 2002] (Step 5 and 6). It finally results in impaired energy production and DNA fragmentation, and eventually ends with cell death [Moroni et al., 2001].
Figure 1.2 The general apoptosis pathway in the injury by cerebral ischemia.
1.1.8 Cellular defence of neuronal death

Under ischemia, the cells would generate a cellular defence mechanism to reduce the
damage or injury. In reducing the generation of free radicals, the following anti-
oxidative enzyme systems are involved [Nordberg and Arner, 2001]:

1. Superoxide dismutase (SOD)

In eukaryotic cells, superoxide anion can be metabolized to hydrogen peroxide by
the SOD isoenzymes. They are copper-zinc superoxide dismutase (CuZnSOD)
present in the cytosol and manganese superoxide dismutase (MnSOD) present in
mitochondria. The reaction catalyzed by SOD provides the source of cellular
hydrogen peroxide as two molecules of superoxide form hydrogen peroxide and
molecular oxygen:

\[
O_2^- + 2H^+ \rightarrow H_2O_2 + O_2
\]

2. Catalase and Glutathione peroxidase (GPx)

Catalase is predominantly present in the peroxisomes and glutathione peroxidase
is present in the cytosol. Both can prevent the formation of hydroxyl radicals by
catalyzing the dismutation of hydrogen peroxide to water and molecular oxygen:

\[
2H_2O_2 \rightarrow O_2 + 2H_2O
\]

On the other hand, the apoptotic process has been found to be inhibited by anti-
apoptotic proteins:

1. B-cell lymphoma/leukaemia-2 (Bcl-2)

The anti-apoptotic effect by upregulating Bcl-2 proteins has been widely
reported [Hutchison et al., 2001; Jacobson and Raff, 1995; Lee et al., 2001;
Martinou et al., 1994; Zhong et al., 1993]. Increased expression of Bcl-2 could
suppress the release of cytochrome c and thereby suppressing the activation of
pro-caspase-9 [Seo et al., 2002] and the subsequent caspase-3 activity [Shimizu et al., 2001; Zhao et al., 2003].

2. Transforming growth factor β-1 (TGFβ-1)

Increasing the expression of TGFβ-1 promotes the phosphorylation of BAD and induces the activation of serine/threonine kinase (Akt), resulting in reduced apoptotic bodies in brain tissues [Chen et al., 2001; Horowitz et al., 2004; Zhu et al., 2002].

3. Serine/threonine kinase (Akt)

The phosphatidylinositol 3-kinase/Serine-threonine kinase (PI3-K/Akt) pathway is recently identified as an anti-apoptotic pathway [Crossthwaite et al., 2002; Neri et al., 2002; Thomas et al., 2002]. Increased expression of activated phosphorylated Akt by the PI3-K/Akt pathway could directly suppress the dimerization of BAD protein with Bcl-XL and the translocation of BAX protein [Andjelkovic et al., 1997; Dudek et al., 1997; Henshall et al., 2002; Kitagawa et al., 1999; Noshita et al., 2001; Ouyang et al., 1999].

1.2 ACUPUNCTURE

1.2.1 Basic introduction

Acupuncture, as a medical modality for various diseases, has been used for more than 2500 years since the early Chinese dynasties. Today acupuncture describes a family of procedures involving the insertion of fine needles into specific anatomical points of the human body by a variety of techniques [Leake and Broderick, 1998; Tai, 2002]. Most of the practices of acupuncture incorporate medical traditions and the
assessment relies on the theory of Traditional Chinese Medicine (TCM). There are four fundamental elements of TCM concept [Ellis et al., 1991; Hopwood et al., 1997]:

1. Vital qi is a basic concept of Chinese medicine, but there is no direct equivalent in Western medicine. It manifests in the skin, in the organs, and permeates every living tissue. It accumulates in the organs and flows primarily in the energy channels and vessels collectively called meridians.

2. Yin-Yang is the balance of two opposing and inseparable forces inside the body. Yin represents the cold, slow, or passive principle, while yang represents the hot, excited, or active principle. Therefore, a disease is regarded as an internal imbalance of yin and yang, leading to the blockage in the flow of vital qi and blood along meridians.

3. In the view of TCM, the major organs are classified into Zhang organs related to Yin or Fu organs related to Yang [Lu et al., 2002]. Zhang organs include Heart, Lung, Liver, Spleen, Kidney, and Pericardium. Fu organs include Small Intestine, Large Intestine, Gallbladder, Stomach, Urinary Bladder, and Triple Burner.

4. Meridians, including channels and connecting vessels, are pathways that carry vital qi, blood, and fluid around the body [Lu et al., 2002]. It ensures the free flow of yin and yang, so that the body is properly nourished. Meridians are described in two main categories: major channels having clearly defined pathways that penetrate deep into the body, and connecting vessels which are their branches. The meridian system is summarized in Figure 1.3 and the nomenclature of meridian defined by the World Health Organization is summarized in Table 1.2 [Ellis et al., 1991].
12 primary channels: connecting internally with the organs and externally with the limbs and joints

12 channels divergences: branching and leading back to the channels

8 extraordinary vessels: acting as reservoirs that regulate the channel system

15 connecting vessels: The main connecting vessels branching from the primary channels, distributing qi and blood and connecting to the the complementary organ-channel system

Minute connecting vessels, branches from the connecting vessels; superficial connecting vessels serving the body surface; blood connecting vessels, small, visible vessels.

Yin:
- Lung channel
- Pericardium channel
- Heart channel
- Spleen channel
- Liver channel
- Kidney channel

Yang:
- Large intestine channel
- Triple burner channel
- Small intestine channel
- Stomach channel
- Gallbladder channel
- Bladder channel

Figure 1.3 Classification of meridian system.
Table 1.2  Nomenclature and code of meridians defined by World Health Organization [Ellis et al., 1991].

<table>
<thead>
<tr>
<th>1. Lung channel</th>
<th>L</th>
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<tbody>
<tr>
<td>2. Large intestine channel</td>
<td>LI</td>
<td>II</td>
<td>LI</td>
</tr>
<tr>
<td>3. Stomach channel</td>
<td>S</td>
<td>III</td>
<td>ST</td>
</tr>
<tr>
<td>4. Spleen channel</td>
<td>Sp</td>
<td>IV</td>
<td>SP</td>
</tr>
<tr>
<td>5. Heart channel</td>
<td>H</td>
<td>V</td>
<td>HE</td>
</tr>
<tr>
<td>6. Small intestine channel</td>
<td>SI</td>
<td>VI</td>
<td>SI</td>
</tr>
<tr>
<td>7. Bladder channel</td>
<td>B</td>
<td>VII</td>
<td>BL</td>
</tr>
<tr>
<td>8. Kidney channel</td>
<td>K</td>
<td>VIII</td>
<td>KI</td>
</tr>
<tr>
<td>9. Pericardium channel</td>
<td>P</td>
<td>IX</td>
<td>PE</td>
</tr>
<tr>
<td>10. Triple burner/Energizer channel</td>
<td>TE</td>
<td>X</td>
<td>TB</td>
</tr>
<tr>
<td>11. Gallbladder channel</td>
<td>G</td>
<td>XI</td>
<td>GB</td>
</tr>
<tr>
<td>12. Liver channel</td>
<td>Liv</td>
<td>XII</td>
<td>LV</td>
</tr>
<tr>
<td>13. Governor vessel</td>
<td>GV</td>
<td>XIII</td>
<td>Du</td>
</tr>
<tr>
<td>14. Conception vessel</td>
<td>CV</td>
<td>XIV</td>
<td>Ren</td>
</tr>
</tbody>
</table>

Standard code is defined by WHO Regional Working Group on the Standardization of Acupuncture Nomenclature.
1.2.2 Characteristics of acupoint

Besides meridians, acupoints or acupuncture points are the important elements of acupuncture. They are located along the skin with the properties of lower electrical impedance, higher electrical potential, and more peripheral nerves than other areas of the skin [Leake and Broderick, 1998; Rosenblatt, 1981]. There are three types of acupoints: Channel points, Extra points and Ashi points [Xiao and Mu, 2000]. Channel points are points pertaining to channels. A total of 361 Channel points are generally acknowledged. Extra points are not pertaining to channels. A total of 50 Extra points are in frequent use in clinical practice. Ashi points refer to those points that can relieve sufferings or pain when pressed. Each acupoint has a general function to reflect the pathological changes for diagnosis and to accept the stimuli for treatment of diseases.

1.2.3 Forms of acupuncture

Acupuncture can be broadly divided into several types: Manual acupuncture, electro-acupuncture (EA), transcutaneous electrical nerve stimulation (TENS), and laser acupuncture.

1. Manual acupuncture

Manual acupuncture involves needle manipulation after insertion of acupuncture needles. Followings are typical methods of needle manipulation [Ellis et al., 1991]:

(a) Lifting and thrusting: After qi has been obtained, the needle is lifted a short distance and then thrust back to the original depth.
(b) Twirling or rotating: When the needle has been inserted to the proper depth and qi has been obtained, the practitioner grasps the handle of the needle between his thumb and forefinger and twists the needle first one way and then the other.

(c) Retaining the needle: In modern practice, needles are often left in place for a period of time ranging from several minutes to two hours depending on the particular condition. This allows for application of other stimulation methods such as warming the needle, electrical stimulation, or intermittent stimulation. Often the needles are retained with little or no additional manipulation.

2. Electroacupuncture (EA)

Electro-acupuncture involves the insertion of acupuncture needles and applies an electrical stimulation to the needles with an EA apparatus after reaching the acupoint called “Deqi” sensation. Usually the EA apparatus are design to deliver variable amplitudes and frequencies of electrical pulses with waveform fixed. The waveform may be symmetric biphasic, alternating or asymmetrical biphasic pulses of varying configurations. The pulse width is commonly of fixed duration (0.2 or 0.4 ms). There are three types of EA currently using [Ellis et al., 1991; Hopwood et al., 1997]:

(a) Low-frequency EA: Frequencies of stimulation between 2 Hz and 4 Hz are used. Such stimulation produces analgesia in normal subjects. To obtain pain relief, a high intensity of stimulation which is sufficient to produce muscle or muscle fibre contractions is recommended.

(b) Low-frequency pulse train: This technique is a mode of stimulation that delivers impulses of 2 Hz with an inner train frequency of about 80 Hz.
Meeting the requirement of muscle fibre contraction is possibly more tolerable with this mode of stimulus.

(c) High-frequency EA: The large diameter sensory fibres are effectively stimulated when a frequency of 80-100 Hz is used at intensities sufficient to produce a comfortable degree of tingling (paraesthesia).

3. Transcutaneous electrical nerve stimulation (TENS)

Transcutaneous electrical nerve stimulation (TENS) is the application of electrical stimulation to the skin via surface electrodes to stimulate afferent nerves for pain relief. There are currently four TENS modes used in clinical practice [Hopwood et al., 1997]:

(a) High-frequency, low-intensity TENS (Conventional TENS): It is the most commonly used mode of TENS. The stimulation parameters are low intensity, and high frequency, typically around 100 Hz. The pulse duration is usually short (50-80 µs). This TENS mode achieves its analgesia primarily by spinal segmental mechanisms (i.e. gating effects).

(b) Low-frequency, high-intensity TENS (Acupuncture-like TENS): The stimulation parameters are low frequency (1-4 Hz), high intensity and long pulse duration (~ 200 µs). The patient will experience paraesthesia and muscle contraction (twitching type) with this mode.

(c) Burst-train TENS: This mode of TENS is really a mixture of conventional and acupuncture-like TENS, in which a baseline low-frequency current is delivered that contains high-frequency trains. The frequency of trains is 1-4 Hz with the internal frequency of the trains around 100 Hz.
(d) Brief, intense TENS: This mode of TENS uses a high frequency (100-150 Hz), long pulse duration (150-250 µs) and highest tolerable intensity for short periods (<15 min).

1.2.4 Acceptance of the use of acupuncture

Over many centuries in history, an alternative medicine has come and gone, yet acupuncture has always continued to grow and its usage has been extended from China to the World. In England, one-third to one-half of the general population is using one or more forms of alternative therapies [Schmidt et al., 2002]. Medical doctors in the Germany and the UK, where acupuncture is ranked as the first selection of alternative therapies, also refer patients to those acupuncture practitioners [Schmidt et al., 2002]. Another survey conducted in the North East Scotland also found that the number of patients using acupuncture increased from 6% to 10% from 1993 to 1999 [Emslie et al., 2002]. However, its safety and effectiveness are always being challenged [Boon, 2002; Ernst, 2002; Walker and Budd, 2002].

1.2.5 Possible mechanism for experimental stroke study

There are several possible mechanisms being postulated in using experimental stroke model:

1. In western medicine, acupuncture is regarded as a type of sensory stimulation that can activate multiple efferent pathways and alter the activity of the neurological system [Wong et al., 1999]. Its effect may therefore be obtained by neuromuscular stimulation. Acupuncture can enhance the functional plasticity of
the brain [Johansson, 2000; Kjendahl et al., 1997; Magusson et al., 1994; Wong et al., 1999]. This improved plasticity was likely due to the acupuncture-influenced intrinsic cortical circuits in the focal damaged brain. Subsequently the brain tissue can modify itself through changes at the cellular level such as the neuronal and glial cell extensions and synapses [Gosman-Hedstrom, 1998].

2. Acupuncture can reduce the blood viscosity, thereby dredging the circulation, decreasing peripheral vascular resistance, improving blood supply to anoxic brain tissue and reducing micro-emboli formation. Thus, it facilitates the recovery of the damaged brain tissue by providing sufficient supply of blood [Ji et al., 1998; Yang et al., 1999].

3. Electro-acupuncture at a low frequency (1-2 Hz) can produce analgesia effect by increasing levels of beta-endorphin levels and adrenocorticotropin (ACTH) in the central nervous system. Also electro-acupuncture produces the modulatory effect by increasing the level of neuropeptides, which is related to the stable, gentle improvement in motor function of patients with stroke [Naeser et al., 1992].

4. Muscle stimulation generated by acupuncture is able to induce the release of transmitters and neuropeptides, and to stimulate trophic factors that enhance recovery from stroke [Magusson et al., 1994]. One of the examples of these transmitters and neuropeptides is basic Fibroblast Growth Factor (bFGF). Applying electro-acupuncture during ischaemia and reperfusion could upregulate the expression of bFGF-like immunoreactivity in the striatum and cortex. Other studies also reported that the intravenous injection of bFGF could protect hippocampal neurons against ischaemia-reperfusion injury by penetrating the blood-brain barrier [Yang et al., 1999]. As a result, electro-acupuncture might be effective in reducing the brain damage and allowing the better recovery of
patients with stroke [Chen et al., 1998; Yang et al., 1999].

5. Acupuncture could enhance the auto-compensatory ability of neurons to protect against excitotoxicity. This mechanism was driven as a two-way alternation of extracellular excitatory and inhibitory amino acids. Acupuncture can improve the elevation of taurine and decrease the rise of aspartate level during ischemia in the rats, therefore triggering the activation of endogenous protection and serving as the electro-acupuncture-stimulated neuro-protection [Zhao et al., 1997].

1.2.6 Current findings from clinical studies of stroke

In mainland China, acupuncture or electroacupuncture has been extensively used for patients with stroke. There are numerous beneficial effects reported and some of the representative studies are introduced in the following. The clinical study conducted by Johanasson et al. (1993) recruited 78 patients with subacute stroke in Lund University Hospital. They were suffering from acute or chronic effects and some even suffering with severe hemiparesis to the level that they could not walk, eat and drink without assistance. In that study, these patients were randomly divided into the control and electro-acupuncture groups, in which the latter group received 30-minute electro-acupuncture twice per week lasting for 10 weeks. Both groups were subjected to the standard stroke rehabilitation program. Assessment of motor function, balance and Barthel Index (BI) were conducted before therapeutic treatment, and at 1 and 3 months after acute stroke. Activities of Daily Life (ADL) were measured after 12 months and the quality of life was measured at 3, 6, 12 months following acute stroke. Significantly better improvement was observed in the acupuncture group in all measurement except the motor function. In 1993, Hu et al. (1993) investigated the
CHAPTER 1: INTRODUCTION

effect of acupuncture treatment on the neurological and functional recovery in 30 patients with acute and first-ever stroke within 36 hours. All patients were randomly assigned to rehabilitation therapy with or without 60-minutes electro-acupuncture, 3 times per week lasting for four weeks. More than 10 scalp and body acupoints were prescribed as main points in the treatment protocol. Scandinavian Stroke Scale (SSS) and Barthel Index (BI) were adopted as the outcome measures. The acupuncture group showed significantly better improvement ($P<0.05$) in the SSS score but not in the BI. Moreover, further statistical analysis according to the subgrouping, the greatest improvement in neurological status was observed in patients with lower neurological score at baseline. Similarly, Kjendahl et al. (1997) investigated functional and motor level of 45 patients with hemiparesis (35 with infarction and 10 with hemorrhage) who suffered first time of stroke with median post-stroke time of 40 days. The intervention group received 30-minutes acupuncture treatment, 3 to 4 times a week lasting for 6 weeks. In addition, both groups received regular physiotherapy or self-administered exercise for 1 to 3 times a week from discharge to 12 months afterwards. Blinded assessor was responsible to evaluate the Motor Assessment Scale (MAS), Sunnaas Index of Activities of Daily Life (ADL) and Nottingham Health Profile (NHP). Results showed that, all the parameters were significantly improved in the intervention group than the control group. These effects could also be observed in patients with cerebral infarction. Si et al. (1998) first confirmed totally 42 patients with cerebral infarction by using computed tomography. The intervention group received electro-acupuncture 5 times a week and continued until patients were discharged from hospital. Both groups received routine drug treatment. Evaluation was done by Chinese Stroke Scale (CSS) which assessed the level of consciousness, extraocular movement, facial palsy, speech, capacity walking, motor shoulder, leg and
hand. The score ranged from 0 to 43, and a higher score indicates greater neurological deficit. The result showed that the motor functions of shoulder, hand and leg of the intervention group was significantly improved than the control group ($P<0.01$).

On contrary, some of the studies reported that there was no beneficial effect observed in patients with brain damage after receiving acupuncture or electro-acupuncture treatment. The following showed some representative example. Gosman-Hedstrom et al. (1998) studied the effect of acupuncture treatment on daily life activities and Quality of Life (QOL) in 104 non-haemorrhagic stroke patients admitted to a Swedish hospital. Patients were suffering from severe paresis to the level that they could not walk, eat, drink, and dress without assistance. Patients were randomly assigned into 3 groups: deep acupuncture, superficial acupuncture and no acupuncture. Superficial acupuncture (Sham) was used to give similar level of attention to the patients as in the deep acupuncture so as to eliminate the psychological impact. Acupuncture was started on 4-10 days after randomization for 20 sessions over 10 weeks. Electrical or manual stimulation was used for 30 minutes. All groups received conventional stroke rehabilitation program. Two blinded occupational therapists were used to evaluate the effects four times during the first year. Outcome measures used were Scandinavian Stroke Scale (SSS), Barthel index (BI), Sunnas ADL, and Nottingham Health Profile (NHP). However, there were no significant difference between the control and the intervention groups. Moreover, Johanasson et al. (2001) recruited 150 patients with stroke onset of 5-10 days in Sweden. These patients were having moderate to severe functional impairment ($\text{BI} \leq 70$), and were unable to accomplish the Nine Hole Peg Test within 60 seconds or to walk for 10 metre without assistance. Patients were
randomly assigned into acupuncture, TENS and control groups. Thirty-minute acupuncture treatment was provided twice per week for 10 weeks and only 9 acupoints were prescribed. The effect was strengthened either by manual or electrical stimulation. In the TENS group, electrical stimulation was delivered via adhesive electrodes over the acupoints to induce visible muscle contraction. In the control group, electrical stimulation below the perception threshold was given. All groups were subjected to the conventional stroke rehabilitation. Blind assessor was used to evaluate the Barthel Index (BI), Notthingham Health Profile (NHP), walking ability and Rivermead Mobility Index at 3 and 12 months. However, there was no significant difference observed among three groups. Recently, Sze et al. (2002) examined whether acupuncture had additional value to post-stroke patients. Chinese patients (totally 106 patients) in Hong Kong within 15 days of stroke onset were recruited. Patients with Barthel Index (BI) <3 or $\geq 15$, haemodynamic instability or dementia were excluded. They were first divided into 2 groups: moderate (BI<11) and severe (BI $\geq 11$), and further subdivided into acupuncture and control groups. 30-minute manual acupuncture treatment without manipulation was provided for 10 weeks in totally 35 sessions. There were 10 body acupoints were prescribed as main acupoints and 6 auxiliary acupoints were also added to allow some flexibility of the treatment protocol. All groups received standard post-stroke rehabilitation program. Blinded assessor performed the evaluation of Fugl-Meyer Assessment of Physical Performance-Motor subsection (FMAM), Barthel Index (BI), Functional Independence Measure (FIM) prior to treatment and at 5 and 10 weeks after treatment. No significant difference was found among all groups as both arms of moderate and severe groups showed similar improvement in motor impairment and disability.
CHAPTER 2

Aim of the study

The acceptance of using acupuncture as a form of the treatment for diseases is increasing. However, the inconsistent outcomes are resulted when using acupuncture or electroacupuncture for the stroke treatment. The scientific basis of the mechanism of acupuncture is still remaining unclear. Therefore, this study is aimed at investigating the effectiveness of the electroacupuncture on attenuating the reactive oxygen species-mediated injury and apoptosis induced by cerebral ischemic-reperfusion injury in adult rats. The time of intervention is also assessed by employing the pre-ischemic EA stimulation prior to the induction of cerebral ischemia and the delayed post-ischemia EA stimulation.
CHAPTER 3

Animal model of transient focal cerebral ischemia

3.1 INTRODUCTION

For a systematic study of the treatment of electro-acupuncture (EA) on cerebral ischemia, it is essential to use a physiologically controlled and reproducible in vivo animal model. The model using non-human primates is undoubtedly the closest one to the ideal model of ischemic stroke [Eklöf and Siesjö, 1972; Nordström and Siesjö, 1987]. However, the rat has been selected in this study because of its several advantages over larger animal species, notably rabbits and gerbils. Firstly, the high homogeneity within strains owing to inbreeding is suitable for the large scale studies for statistical analysis [Cocchetto and Bjornsson, 1983; Ginsberg and Busto, 1989]. Secondly, the close resemblance of the cerebrovascular anatomy and physiology of rats to that of humans share the same level of significance [Ginsberg and Busto, 1989; Yamori et al., 1976]. Finally, the lower cost of rats and the expenses for surgical operation are considered [Chen et al., 1986; Cocchetto and Bjornsson, 1983; Ginsberg and Busto, 1989].

Several models of cerebral ischemia have been developed in the rat. It can be generally classified by topography as global or focal ischemia [Chen et al., 1986; Ginsberg and Busto, 1989]. Under focal ischemia, it can be further classified by chronology as transient (reversible) or permanent (irreversible) [Chen et al., 1986; Ginsberg and Busto, 1989; Murphy, 2000]. Clinical findings reported that more than 75% of stroke patients were suffered from the occlusion of middle cerebral artery.
that was the transient focal cerebral ischemia. This animal model used in this study has gained an increasing acceptance owing to their relevance to the human clinical settings [Garcia, 1984; Molinari and Laurent, 1976]. Some of the experimental stroke studies were limited to the acute phase (within 24 hours), in which the surgical procedures were established by Tamura et al. (1981). They ligated the MCA at the proximal region close to the olfactory tract to the inferior cerebral vein, resulting in 100% infarction rate [Bederson et al., 1986]. This operation was technically difficult and sufficiently invasive, so that the survival of hours of rats limited it to acute-phase experiment. On the other hand, this study employed the procedures established by Chen et al. (1986) that was surgically less demanding and allowed high survival rate of rats. As the ischemic-reperfusion injury to neurons is progressive and time-dependent [Garcia, 1974; Garcia, 1983; Waltz et al., 1972], it is attempted to investigate the impact of injury at the sub-acute phase (from 1 to 5 days following the cerebral ischemia).

To confirm the establishment of transient focal ischemia, the ischemic rats were evaluated on the aspects of physiological responses, neurological deficits, and biochemical changes at cellular level. The arterial blood was obtained to measure physiological parameters, including partial pressure of oxygen (paO$_2$) and carbon dioxide (paCO$_2$), and pH. The neurological deficits were assessed by the motor functions according to the widely used examination scheme developed by Bederson et al. (1986). In the biochemical level, the potential damage triggered by nitric oxide was determined by total activity of nitric oxide synthases (NOS) and expressions of three isoforms (neuronal NOS, nNOS; inducible NOS, iNOS; and endothelial NOS,
eNOS). On the other hand, the anti-oxidant activities by free radical scavengers, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were also measured. As a consequence of these biochemical reactions, the level of lipid peroxidation was determined by quantifying the amount of malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE).

3.2 MATERIALS AND METHODS

3.2.1 Approval of animal experiment

All experimental procedures described below were approved by the Animal Subject Ethics Sub-committee (ASESC project number: 99/7) of The Hong Kong Polytechnic University. To comply with the statutory requirements stated in the Animals (Control of Experiments) Ordinance (Chapter 340) of Hong Kong, a license for the use of animals for this study was obtained (Licence number: DH/KRO/P07/01/3).

3.2.2 Chemicals

Unless stated otherwise, all materials were of analytical grade and obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Protein concentration was determined by using protein assay as described by Bradford (1976) (Bio-Rad Laboratories, Hercules, CA, USA).

3.2.3 Experimental design

A total of 70 Sprague-Dawley rats (male, 350-380 g) were used for the measurement of physiological parameters and evaluation of neurological deficits (n=22), and the determination of timely response of various biochemical factors following transient
focal cerebral ischemia (n=48). In the first experiment, rats were divided into two
groups for collecting arterial blood samples before and after the onset of cerebral
ischemia. The behaviour and motor functions of these rats were observed. In the
second experiment, rats were evenly distributed into six groups: Normal group (rats
without induced cerebral ischemia) and Ischemia groups of each group having eight
rats (rats induced with cerebral ischemia and harvested at post-ischemia day 1 to day
5). Rats in both experiments were anesthetized with intraperitoneal injection of
ketamine (70 mg/kg, Alfasan, Holland) and xylazine (7 mg/kg, Alfasan, Holland)
before induction of cerebral ischemia. A second dose of ketamine (30 mg/kg) and
xylazine (3 mg/kg) was given at 40-45 min of cerebral ischemia.

3.2.4 Production of transient focal cerebral ischemia

Transient focal cerebral ischemia was induced by occlusion of right middle cerebral
artery (MCA) for 1 hr followed by reperfusion as described by Chen and co-workers
[Chen et al., 1986] with modifications [Leung et al., 2002; Siu et al., 2004]. A 2-cm
scalp incision was made at the midpoint between the right eye and the right ear. The
temporalis muscle was separated in the plane of its fiber bundles to expose the
zygoma and squamosal bone. At the anterior junction of the zygoma and the
squamosal bone, a 5 mm x 5 mm burr hole was made using a saline-cooled electric
drill (Mototoool; Dremel, Denver, Colo, USA). The dura mater was carefully pierced
with a scalpel. Exposed MCA was occluded by the surgical clip for 1 h and was
released for reperfusion. A small piece of absorbent pack was placed to cover the
exposed MCA. Temporalis muscle and overlying skin were allowed to suture
separately. During the surgical procedures, rectal temperature of rats was constantly
monitored and maintained at about 37°C with an overhead lamp. After recovery from
the experimental surgery procedures, all rats were allowed to recover at ambient temperature (21-23°C) with food and water made available *ad libitum* until harvest. Selected photographs taken from surgical operations of cerebral ischemia were shown in Figure 3.1.

### 3.2.5 Measurement of physiological parameters

Blood samples were obtained from femoral artery 15 min before and after the onset of MCA occlusion (Pre-ischemia, n=11; and Post-ischemia, n=11; respectively). A 1-ml arterial sampling syringe (PICO™50, Radiometer, Copenhagen, Denmark) containing lyophilised compensated heparin (50 IU) was employed to minimize the cation binding to the sample prior to analysis. Physiological parameters, blood gases in terms of arterial partial pressure of oxygen (paO₂) and carbon dioxide (paCO₂), and pH were determined using a blood gas analyzer (ABL505, Radiometer, Copenhagen, Denmark).

### 3.2.6 Neurological examination

Neurological status of each ischemic rat was carefully evaluated at 1 hour, 4 hours and 24 hours after induction of cerebral ischemia. According to the Neurological Examination Grading System as shown in Table 3.1, a grading scale of 0-3 was used to assess the effects of occlusion [Bederson *et al.*, 1986]. Rats were held gently by the tail, suspended 20-30 cm above the working bench, and observed for forelimb flexion. Rats able to extend both forelimbs toward the bench were assigned Normal (Grade 0). Rats consistently flexed the forelimb contralateral to the injured hemisphere were classified as Moderate (Grade 1). Rats were then placed on a large clean towel that could be gripped firmly by their claws. With the tail held by hand, a
force was gently applied on the lateral side behind shoulder until forelimbs slid several centimeters. Also rats were allowed to move for observing circling behaviour. Normal (Grade 0) or Moderate (Grade 1) rats resisted sliding equally in both directions and showed no circling in walking. Severe (Grade 2) rats showed reduced resistance to lateral push toward the paretic side and no circling. Rats with reduced resistance to lateral push and circling movements were designated as Severe (Grade 3).

### 3.2.7 Primary and secondary antibodies

After SDS-PAGE, proteins of interest were electro-blotted onto nitrocellulose membranes and probed with specific antibodies. Isoforms of NOS were identified by using rabbit polyclonal antibodies directed specifically against nNOS, iNOS, and eNOS, respectively (Calbiochem, San Diego, CA, USA; dilution 1:200). Mouse monoclonal antibodies against β-actin were obtained from Sigma Chemical Co. (A5441; dilution 1:2000). Secondary antibodies conjugated with horseradish peroxidase (HRP) directed against rabbit IgG and mouse IgG were obtained from Pierce Chemical Co. (Rockford, IL, USA) and used at a dilution of 1:1000 and 1:5000, respectively.
Figure 3.1 Photographs taken from the surgical operations of cerebral ischemia by occluding middle cerebral artery (MCA).
Table 3.1  Neurological Examination Grading System

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No observable deficit</td>
</tr>
<tr>
<td>1</td>
<td>Forelimb flexion</td>
</tr>
<tr>
<td>2</td>
<td>Decreased resistance to lateral push and forelimb flexion, without circling</td>
</tr>
<tr>
<td>3</td>
<td>Same behaviour as Grade 2, with circling</td>
</tr>
</tbody>
</table>
3.2.8 Assay of NOS activity by Griess reaction

NOS activity was determined by monitoring the total amount of NO produced from L-arginine by Griess reaction, with the use of a colorimetric assay kit from Cayman (San Diego, CA, USA) [Green et al., 1982]. Principle of reactions of assay was shown in Reaction 3.1 to Equation 3.3 [Nims et al., 1995].

\[
\text{Nitrate reductase} \rightarrow \text{Nitrite} \\
\text{Sulfanilamide} \rightarrow \text{Azo product } \lambda_{\text{max}}: 540 \text{ nm}
\]

Rats were anesthetized and transcardially perfused with 0.38% sodium citrate in saline. The right brain was harvested and weighed before homogenized mechanically with an Ultra-Turrax T25 homogenizer in 2 volumes of 20 mM Tris-HCl (pH 7.4), 7.28 mM AEBSF, 5.6 µM aprotinin, 147 µM leupeptin, 252 µM bestatin, 105 µM pepstatin A, 98 µM E-64 (Protease inhibitor cocktail, P8340, Sigma). The homogenate was centrifuged at 10,000 x g for 30 min at 4°C. Supernatant (50 µl) was incubated with 20 mM Tris-HCl (pH 7.4), 10 mM L-arginine, 1 mM NADPH, 4 µM BH₄, 4 µM FAD, 4 µM FMN, 1 mM CaCl₂ and 5 U calmodulin for 2 h at 37°C. The reaction product was collected and boiled at 100°C for 10 min, followed by
centrifugation at 10,000 x g for 20 min. Nitrate standards and reaction products (80 µl each) were added to a microtiter plate, followed by the addition of 20 mU nitrate reductase and 10 µl of 2 mM working NADH reagent. The plate was incubated for 2 h at room temperature without shaking. Subsequently, 50 µl of sulfanilamide (ρ-aminobenzenesulfonamide) in 3 N HCl was added with gentle shaking. N-(1-Naphthyl)ethylenediamine dihydrochloride (50 µl) was immediately added and the plate was shaken for 10 min at room temperature. The absorbance of both samples and standards were measured at 540 nm using a microtiter plate reader (Winooski, Vermont, USA). The specific activity of NOS was determined as pmol nitrate produced per h per mg protein.

3.2.9 Identification of NOS isoforms expressed by western blotting

Brain homogenate (200 µg) was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% separating gel with 5% stacking gel in a Mini-Protean II Dual Slab Cell (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis for 5 h at 70 V, the separating gel was electro-blotted at 25 V onto a 0.22 µm nitrocellulose membrane overnight. Each membrane was rinsed three times with 1X working Tris-buffered saline-Tween (1X TBS-T: 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20). Washed membranes were blocked with 5% skimmed milk in TBS-T for 2 h at room temperature. The protein blots were individually probed with rabbit IgG raised against rat either nNOS, eNOS or iNOS overnight at 4ºC. Subsequently, the blots were washed six times (5 min each) with 1X TBS-T followed by incubation with anti-rabbit IgG HRP-conjugated antibodies for 2 h with gentle shaking. Afterwards, these blots were washed six times with TBS-T for 5 min each. All NOS isoforms were detected by reacting with Supersignal® West Pico
Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL). Chemiluminescence released was captured by a UVP-Chemi System (UVP, Inc., Upland, CA, USA). The amount of each isoform was quantified by the Lab-Works™ Image Acquisition and Analysis Software (UVP, Inc., Upland, CA, USA). The expression the unit was defined as pg NOS isoform per mg protein of homogenate. Selected blots were re-probed with β-actin as internal control. These blots used mouse monoclonal antibody against β-actin as primary antibody and anti-mouse IgG HRP-conjugated antibodies as the secondary antibody. The detection method was the same as that previously described for NOS isoform.

3.2.10 Assay of total SOD activity

Measurement method of total SOD activity followed the superoxide-driven auto-oxidation of tetracyclic catechol (5,6,6a,11b-tetrahydro-3,9,10-trihydrobenzo[c]fluorene) in alkaline condition to produce a chromophore with maximum absorbance at 525 nm [Nebot et al., 1993] (Calbiochem, San Diego, CA, USA). Reactions of the assay were shown as below:
Brain homogenate was prepared as described in Section 3.2.8. The homogenate was centrifuged at 3,000 x g for 10 min at 4°C. The spectrophotometer for the time scan was set at 525±2 nm (Model U-2000, Hitachi, Japan). Directly to each cuvette of control blank (deionized water) and sample, a 900 µl of 50 mM 2-amino-2-methyl-1,3-propanediol containing 3.3 mM boric acid and 0.11 mM DTPA, pH 8.8 (at 37°C) was added and pre-warmed at 37 ºC for 3 min. Deionized water and brain homogenate (40 µl each) were added to cuvettes and mixed gently. Immediately a 30 µl of 33.3 mM 1,4,6-trimethyl-2-vinylpyridinium trifluomethanesulfonate in 1 mM HCl was added to each cuvette was added, followed by incubated at 37°C for 1 min. Then 30 µl of 0.66 mM tetracyclic catechol in 32 mM HCl, 0.5 mM diethylenetriaminepentaacetic acid and 2.5% ethanol was added. After briefly mixed, the absorbance of products at 525 nm was measured spectrophotometrically for 5 min. The linearly increasing absorbance represented the rate of activity in the presence (Vs) and the absence (Vc) of SOD in samples and control blank, respectively. The total SOD activity was the relative activity derived from the ratio of Vs to Vc. Activity unit was defined as the activity that doubles the auto-oxidation rate of the control blank.

3.2.11 Assay of GPx activity

A coupled enzyme method was employed for determining GPx activity in which the consumption of reduced NADPH was monitored [Paglia and Valentine, 1967]. Reduced NAPDH was used for the regeneration of reduced glutathione (GSH) by glutathione reductase that was essential for measuring GPx activity (Calbiochem, San Diego, CA, USA). The assay did not monitor the activity of glutathione S-transferase
(GST) which catalyzes the denitration of organic nitrates and denitrosation of nitrosoguanidinium compounds [Kuo et al., 2002].

\[
\begin{align*}
\text{Equation (3.4)} & \quad R-O-O-H + 2 \text{GSH} \rightarrow R-O-H + 2 \text{GSSG} + \text{H}_2\text{O} \\
& \quad \text{Glutathione peroxidase} \\
\end{align*}
\]

A 20% (w/v) brain homogenate was prepared as described in Section 3.2.8 and the supernatant was obtained after centrifugation at 10,000 \( \times \) g for 20 min at 4°C. The spectrophotometer for the time scan was set at 340 nm (Model U-2000, Hitachi, Japan). In each curvette, a 350 \( \mu \)l of assay buffer and 350 \( \mu \)l of working NADPH reagent were added to give an assay condition (1 mM GSH, 0.2 mM NADPH, 0.22 mM tert-butyl hydroperoxide, 0.4 unit of glutathione reductase, pH 7.6). Deionized water and supernatant (each 70 \( \mu \)l) was added to cuvettes of control blank and sample, respectively, and mixed by inverting. The absorbance was continuously recorded at 340 nm for 3 min for NADPH consumed for the regeneration of GSH by glutathione reductase. The linear portion of the decreasing absorbance represented the GPx activity. The activity unit was expressed as nmol NADPH oxidized per min per mg protein.

3.2.12 Lipid peroxidation (LPX) assay

Malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE) were determined by employing a colorimetric assay as described by Erdelmeier and co-workers (1998).
(Calbiochem, San Diego, CA, USA). The method was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and 4-HNE. One molecule of either MDA or 4-HNE reacted with 2 molecules of chromogenic reagent to yield a stable chromophore with maximal absorbance at 586 nm.

![Chemical Diagram]

The brain homogenate (10% w/v) was produced as described in Section 3.2.8 and supernatant was obtained after centrifugation at 3,000 x g for 10 min at 4°C. Each sample mixture was made by 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 both MDA and 4-HNE, sample mixture was added with 75 µl of 15.4 M methanesulfonic acid and incubated at 45°C for 40 min. To assay for the quantity of MDA only, sample mixture was added with 75 µl of 12 N HCl and incubated at 45°C for 1 h. After incubation, samples were cooled on ice. The absorbance was read at 586 nm using a microtiter plate reader (Winooski, Vermont, USA). The LPX unit was defined as [MDA] (µM) per mg homogenate protein or [MDA + 4-HNE] (µM) per mg homogenate protein.

3.2.13 Statistical analysis

Data were expressed as mean ± standard deviation. The statistical comparisons of physiological parameters (paCO₂, paO₂, pH) were performed using the paired t test
(SPSS version 11.0, Chicago, IL, USA). Comparisons of Ischemic groups at different days of cerebral ischemia to Normal group were made using an analysis of variance (ANOVA) followed by post-hoc protected least-significant difference (LSD) test. In all instances, it was considered to be statistically significant when $P<0.05$.

3.3. RESULTS

3.3.1. Physiological parameters

The physiological data obtained before and during transient focal cerebral ischemia are shown in Table 3.2. Values of the parameters measured were comparable to those reported by others previously [Hata et al., 1998; Leker et al., 2001; Siu et al., 2004]. The difference between paO$_2$ measured before (105.0 ± 6.7 mmHg) and after (107.3 ± 4.5 mmHg) ischemia was not statistically significant ($t=0.857$, degrees of freedom (d.f.)=10, $a>0.05$). The paCO$_2$ measured before ischemia, 42.5 ± 3.1 mmHg, was only slightly lower than that determined after MCA occlusion, 43.8 ± 3.8 ($t=1.017$, d.f.=10, $a>0.05$). In addition, there was no significant difference between blood pH measured before (7.239 ± 0.037) and after (7.238 ± 0.025) cerebral ischemia. Seemingly the use of ketamine-xylazine would not alter these parameters that were parallel to the observations previously reported [Ridenour et al., 1991; Yang et al., 2002]. These findings suggested that the experimental procedures adopted in the present study did not cause acidosis and the observed changes in cerebral tissues were likely due to the ischemia-reperfusion challenge [Siu et al., 2004].
Table 3.2  Physiological data from rats subjected to transient focal cerebral ischemia.

<table>
<thead>
<tr>
<th></th>
<th>paO₂ (mmHg)</th>
<th>paCO₂ (mmHg)</th>
<th>pH (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-ischemia (n= 11)</td>
<td>105.0 ± 6.7</td>
<td>42.5 ± 3.1</td>
<td>7.239 ± 0.037</td>
</tr>
<tr>
<td>Post-ischemia (n=11)</td>
<td>107.3 ± 4.5</td>
<td>43.8 ± 3.6</td>
<td>7.238 ± 0.025</td>
</tr>
</tbody>
</table>

Data (mean ± standard deviations) obtained from arterial blood samples in rats at pre-ischemia 15 min and after 15 min of cerebral ischemia. Abbreviations: paO₂, partial oxygen pressure; paCO₂, partial carbon dioxide pressure.
3.3.2 Neurological examination in motor function

Motor function of 11 rats was examined after hours of induction of cerebral ischemia and results obtained were shown in Table 3.3. According to the Neurological Examination Grading System, 10 out of 11 rats (91%) were classified into Grade 2 (Moderate) after 1 hour of cerebral ischemia. At 4 hours after cerebral ischemia, percentage of rats with limited forelimb flexion (Grade 2) was dropped to 55% (i.e. 6 rats). Another 4 rats did not show any motor dysfunction. Afterwards no motor dysfunction in rats was observed at 24 hours of cerebral ischemia. The observations suggested that the induction method of cerebral ischemia used in this study was only able to induce a temporary, moderate motor dysfunction.

3.3.3 Total NOS activity and expressions of isoforms

The right brains of both Normal group and Ischemia group of rats were taken for the measurement of total NOS activity and the expression of NOS isoforms. Total NOS activity was determined using Griess reaction that measured the total amount of nitric oxide produced from L-arginine in the presence of Ca$^{2+}$, FAD, FMN, NAPDH, and calmodulin. The results are obtained in Figure 3.2. The Normal group showed the total NOS activity of 290.43 ± 43.16 pmol nitrate produced per hr per mg protein. After reperfusion for 1 and 2 days, the total NOS activity of the Ischemia group was still comparable to that of the Normal group. However, the total NOS activity of the Ischemia group was increasing since 3 days after the induction of cerebral ischemia (P<0.05). This significant increase of the total NOS activity was maintained up to post-ischemia day 5 as measured. The highest activity of the Ischemia group (569.67 ± 68.66 nmol nitrate/hr/mg protein) was found at post-ischemia day 4.
Table 3.3 Examination of motor function of rats after hours of induction of cerebral ischemia.

<table>
<thead>
<tr>
<th>Time after induction of cerebral ischemia</th>
<th>Normal – Grade 0</th>
<th>Moderate – Grade 1</th>
<th>Severe – Grade 2</th>
<th>Severe – Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4 hours</td>
<td>5</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24 hours</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Classes included: Normal (Grade 0), no observable deficit; Moderate (Grade 1), limited forelimb flexion; Severe (Grade 2), decreased resistance to lateral push and no circling in walking; and Severe (Grade 3), decreased resistance to lateral push and circling in walking.
Figure 3.2 Total nitric oxide synthase (NOS) activity measured in the Normal group and the Ischemia group at 1-5 days of cerebral ischemia. Normal group (n=8) did not receive any surgical operation, while Ischemia group harvested at post-ischemia 1-5 days received 1-hour MCA occlusion followed by reperfusion (n=8 per each day). Total NOS activity was determined by monitoring the total amount of NO produced from L-arginine by Griess reaction. Activity unit was defined as pmol nitrate produced per hr per mg protein. Values were mean±S.D. ANOVA followed by post-hoc protected LSD test was used for statistical significance. Symbol (a) denoted the significance difference of Ischemia group to Normal group (P<0.05).
After cerebral ischemia, the expression of NOS isoforms was found to be time-specific. Protein homogenate was resolved by SDS-PAGE and individually reacted with antibodies specifically directed nNOS, iNOS or eNOS. Expression patterns in protein blots and their corresponding amounts are obtained in the Figure 3.3. In the Normal group, the relative amounts of expressed isoforms were 222.29 ± 7.03 pg nNOS per mg protein, 75.66 ± 9.76 pg iNOS per mg protein, and 119.34 ± 4.97 pg eNOS per mg protein. One day after 1-hour MCA occlusion and reperfusion, the expression levels of nNOS and iNOS were maintained as comparable to that of the Normal group. From post-ischemia day 2 to day 3, nNOS expression was gradually increasing and reached a plateau at post-ischemia day 4 (with 468.28 ± 48.40 pg nNOS per mg protein) (P<0.05). However, expression levels of iNOS were slightly reduced in day 2 and 3 (P<0.05). Subsequently, in comparison to the Normal group, a two-fold increase of iNOS expression (177.32 ± 22.6 pg iNOS per mg protein) was observed since post-ischemia day 4 (P<0.05). Unlike nNOS and iNOS, eNOS detected was firstly varied from post-ischemia day 1 to day 3 Expression of eNOS was also enhanced by two-fold since post-ischemia day 4 and maintained at about 236.48 ± 36.21 pg eNOS per mg protein (P<0.05). Except in day 3, the expression pattern of isoforms was in line with the total NOS activity measured in above.

### 3.3.4 Total SOD activity and GPx activity

To determine an intracellular ability to remove free radicals, brain homogenates of both Normal group and Ischemia group were taken for measuring the SOD and GPx activities. Their results are obtained in Figure 3.4 and Figure 3.5, respectively. The total SOD activity was measured as a relative activity measured under the assay condition in the presence of SOD and in the absence of SOD. According to Figure
3.4, the SOD activity of the Normal group was found at $0.92 \pm 0.11$. After 1-hour MCA occlusion, the total SOD activity was increased by 30% to $1.20 \pm 0.08$ on post-ischemia day 1, and by 25% to $1.15 \pm 0.2$ on post-ischemia day 2 (P<0.05). This temporarily upregulated activity was restored to the level of the Normal group ($0.92 \pm 0.11$) after post-ischemia day 3. GPx activity was determined from the NADPH consumption for the regeneration of reduced glutathione (GSH) that was a cofactor of GPx to reduce the organic hydroperoxide in the assay. According to the results shown in Figure 3.5, the GPx activity of the Normal group was detected at $281.86 \pm 7.39$ nmol NADPH oxidized per min per mg protein. A transient increase of GPx activity ($302.86 \pm 5.33$ nmol NADPH/min/mg protein) was only observed at one day after induction of cerebral ischemia (P<0.05). No difference of GPX activity was observed between the Normal group and the Ischemia group on the following days.

### 3.3.5 Lipid peroxidation

Level of lipid peroxidation was determined from the concentrations of its products, MDA and 4-HNE, which are obtained in Figure 3.6(A) and Figure 3.6(B), respectively. In the Normal group, the basal concentrations of lipid peroxidation were $26.19 \pm 4.2 \mu M$ MDA per mg protein and $82.62 \pm 3.85 \mu M$ MDA+4-HNE per mg protein. The amount of lipid peroxidation products in the Ischemia group in all days were significantly greater than those in the Normal group (P<0.05). At post-ischemia day 1, the amount of lipid peroxidation was greatly increased by two-fold to give $53.59 \pm 3.84 \mu M$ MDA per mg protein (P<0.05) and by 50% to give $121.56 \pm 8.79 \mu M$ MDA+4-HNE per mg protein (P<0.05). The amount of lipid peroxidation products was consistently increasing until post-ischemia day 4 when a plateau was reached.
Figure 3.3  Protein blots of NOS isoforms measured in the Normal group and Ischemia group, with β-actin as an internal control: (A) Neuronal NOS (nNOS), (C) Inducible NOS (iNOS) and (E) Endothelial NOS (eNOS). Amounts of nNOS, iNOS and eNOS were quantified and obtained in (B), (D), and (F), respectively. The unit was expressed as pg NOS isoform per mg protein. Values (mean±S.D., n=8 per group) were analyzed by ANOVA followed by post-hoc LSD test. Symbol (a) denoted the significant difference to the Normal group (\(P<0.05\)).
Figure 3.4 Total superoxide dismutase (SOD) activity measured in the Normal group and Ischemia group at 1-5 days of cerebral ischemia. Total SOD activity was determined by quantifying the reaction products in superoxide-driven auto-oxidation of tetracyclic catechol. One activity unit was defined as an activity doubling the auto-oxidation rate of tetracyclic catechol. Data (mean±S.D., n=8 per group) was analysed by ANOVA with post-hoc LSD test. Symbol (a) indicated the significant difference to Normal group ($P<0.05$).
Figure 3.5  Glutathione peroxidase (GPx) activity measured in the Normal group and Ischemia group after 1-hour MCAO followed by reperfusion for 1-5 days. GPx activity was determined from a coupled enzyme assay in which the consumption of reduced NAPDH was monitored. The activity unit was expressed as nmol NADPH oxidized per min per mg protein. The data was mean±S.D., n=8 in each group. ANOVA followed by post-hoc protected LSD test was used for statistical significance. Symbol (a) denoted the significant difference between the Normal group and Ischemia group ($P<0.05$).
Figure 3.6  Levels of lipid peroxidation products measured in the Normal group and Ischemia group: (A) only malondialdehyde (MDA) and (B) MDA + 4-HNE (4-hydroxy-2(E)-nonenal). The concentration was determined by reacting with the chromogenic reagent, N-methyl-2-phenylindole. LPX was defined as [MDA] (µM) or [MDA + 4-HNE] (µM) per mg protein. Values (mean±S.D., n=8 in each group) were analyzed by ANOVA followed by post-hoc LSD test. Symbol (a) represented the significant difference to Normal group (P<0.05).
3.4 DISCUSSION

Clinically, patients survived from stroke are usually associated with motor dysfunction or even worse with hemiplegia. Such a motor functional inability is currently taken as an index of neurological deficits induced stroke. This study therefore used the Neurological Examination Grading System [Bederson et al., 1986] which assesses the functional behaviour of ischemic rats so to determine the neurological deficits. However, in this study, only a temporary, moderate motor dysfunction was observed in rats after 4 hours of cerebral ischemia and none was found after 24 hours. Although Bederson et al. (1986) postulated a linear relationship of percentage of infarction to neurologic grade in rats after assessment at 24 hours of cerebral ischemia, a number of studies reported that no motor dysfunction was found [Chen et al., 1986; Yoshimine and Yanagihara, 1983]. In the rat and the gerbil, no impairment of motor function occurred even though a large cortical infarct was produced [Chen et al., 1986; Fassbender et al., 2000; Garcia, 1984; Yoshimine and Yanagihara, 1983]. Such a finding was probably because the primary motor cortex in the rat was located medially and is spared [Hall and Lindholm, 1974], or the primary motor cortex may be needed only for complex motor performance and subcortical nuclei may suffice for ambulation [Chen et al., 1986]. Moreover, the transient motor impairment was impossible due to the protection by ketamine. In contrast to the neuroprotection by barbiturates [Hoff et al., 1975; Smith et al., 1974; Yatsu et al., 1972], ketamine would not exert any effect on brain tissues [Garcia, 1984; Molinari and Laurent, 1976; Takeshita et al., 1972]. In addition, other neurological dysfunction such as respiratory abnormalities, seizures and impairment of consciousness were also difficult to assess in small laboratory animals such as the rat and the gerbil [Garcia, 1984; Waltz, 1979]. Therefore, the lack of motor impairment
eliminated behaviour analysis or grading of neurological deficits as the index of neuronal severity and the ischemic model was thus evaluated by the biochemical parameters.

Upon the cessation of blood flow and deprivation of oxygen and nutrients, a series of neuronal injuries, including the elevation of extracellular glutamate and its receptors, and the increase in intracellular calcium content, resulted in the generation of free radicals [Clavier et al., 1994; Samdani et al., 1997; Zhang et al., 1995]. Nitric oxide (NO), which can be produced within minutes, is one of the first reacting free radicals generated during cerebral ischemia [Iadecola, 1997; Kader et al., 1993]. NO is generated by a two-step conversion of L-arginine at the guanidine group and is catalyzed by an enzyme called nitric oxide synthase (NOS) [Moncada et al., 1991]. Three NOS isoforms: neuronal NOS (nNOS) [Bredt and Snyder, 1990; Schmidt et al., 1991], inducible NOS (iNOS) [Yui et al., 1991] and endothelial NOS (eNOS) [Pollock et al., 1991] have been identified. The increase in NO production is usually accompanied by upregulation of both NOS activity and protein expression of NOS isoforms [Holtz et al., 2001]. In this study, therefore, the total NOS activity was determined by the Griess Reaction and protein expression of three isoforms was quantified by western blotting. Our results showed that the total NOS activity of ischemia-reperfused rat brains was not significantly different from that of the Normal group until day 3 after induction of cerebral ischemia. The enhanced NOS activity reached the plateau since day 4 of cerebral ischemia. The results suggested that the animal model used in this study could markedly induce the NO production at the sub-acute stage (after 24 hours) that was in line with the observations reported [Fassbender et al., 2000; Zhu et al., 2002]. Correspondingly the protein expression of
each isoform was consistently increased and reached the maximum at day 4 after cerebral ischemia. Induction of NOS expression after acute stage and their implication in neuronal injury had been extensively reported. Leker et al. (2001) reported that the number of nNOS-expressed cells in cortical slices was peaked at 24-48 hours after transient cerebral ischemia. The excessive nNOS-mediated NO production triggered the glutamate-mediated neurotoxicity that contributed to the metabolic deterioration [Iadecola, 1997; Samdani et al., 1997]. In addition to the actions by nNOS, iNOS was another isoform of NOS contributed to the majority of neurotoxicity. Grandati et al. (1997) observed that iNOS begun to increase after 3 days of ischemia-reperfusion that was later peaked at 7 days and returned to baseline at 10 days. Fassbender et al. (2000) found that the increased iNOS expression at post-ischemia day 2 after 2-hour transient MCAO. Lerouet et al. (2002) reported that iNOS activities in both cortex and striatum region were extensively increased after 48 hours of cerebral ischemia. The late onset of iNOS-induced expression was likely due to subacute glial activation and intracerebral migration of macrophages after 2 days at injury site [Garcia et al., 1994; Kochanek and Hallenbeck, 1992]. Usually the amount of NO generation by induced iNOS at subacute stage was the greatest [Grandati et al., 1997; Iadecola et al., 1995] and could promote the progressive cerebral infarction in both ischemic core and ischemic penumbra region [Ashwal et al., 1998; Iadecola, 1997; Leker et al., 2001; Samdani et al., 1997; Zhang et al., 1993]. On the other hand, the continuous increase in eNOS expression observed in this study was similar to other findings previously reported. In the study by Leker et al. (2001), eNOS expression was continued to increase up to 7 days after MCA occlusion. The induced eNOS proteins were able to improve blood flow in situ [Leker et al., 2001; Wei et al., 1999; Zhang et al., 1993]. However, at the subacute stage of cerebral ischemia, the
cerebroprotective effect of eNOS was insufficient to encounter the neurotoxic effect of nNOS and iNOS [Iadecola, 1997], thereby resulting in exacerbated cerebral infarction and increased levels of lipid peroxidation.

Apart from nitric oxide, other reactive oxygen species, particularly superoxide anion (O$_2$•$^-$) and hydrogen peroxide (H$_2$O$_2$), can cause damages to membrane phospholipids, proteins and DNA [Michels et al., 1994]. These species can be effectively scavenged by the coupling actions of antioxidant enzymes, namely superoxide dismutase (SOD) and glutathione peroxidase (GPx). SOD converts O$_2$•$^-$ to H$_2$O$_2$ and GPx converts the generated H$_2$O$_2$ into water and molecular oxygen [Crack et al., 2003; Michels et al., 1994; Okabe et al., 1998]. However, the sudden burst of ROS after cerebral ischemia-reperfusion causes these antioxidant enzymes impaired, resulting in the triggered lipid peroxidation and the exacerbated cerebral infarction. In this study, their enzymatic activities were examined after transient focal cerebral ischemia. Total SOD activity measured in this study represents activities of both copper-zinc SOD (CuZnSOD) in cytosolic fraction and manganese SOD (MnSOD) in mitochondrial fraction. Our results found that the total SOD activity was increased at day 1 and this increase was only maintained for 2 days after cerebral ischemia. This observation agreed with previous studies that both MnSOD and CuZnSOD could be induced by transient focal cerebral ischemia [Kato et al., 1995; Ohtsuki et al., 1993]. It was found that the upregulation started as early as 4 hour and reached the maximum at day 1 after ischemia [Kato et al., 1995]. The increased SOD activity may play a protective role in the development of ischemic tolerance and the survival of neurons after ischemia [Keller et al., 1998]. Similarly the increased GPx activity at post-ischemia day 1 was observed in our study that paralleled with those previously reported [Keller et al., 1998]. The associated increase in GPx activity suggested a
compensatory response to increased H$_2$O$_2$ levels produced by total SOD activity [Keller et al., 1998]. However, an exacerbated production of MDA and 4-HNE was resulted in ischemic rats at the first two days after cerebral ischemia. Kondo et al. (1997) had reported that levels of MDA and 4-HNE were increased in the frontal cortex and hippocampus within 1 day after transient focal cerebral ischemia. Greater lipid peroxidation was probably because the ischemia-induced activities of SOD and GPx were not effective enough to prevent actions of reactive oxygen species [Siu et al., 2002], or accumulated H$_2$O$_2$ was produced from excess conversion of O$_2^-$ to H$_2$O$_2$ by SOD without a concomitant increase of GPx [Trépanier et al., 1996; Weisbrot-Lefkowitz et al., 1998].

In this study, after day 3 of cerebral ischemia, the enormous production of NO and declined activities of SOD and GPx resulted in the exacerbation of lipid peroxidation. This observation agreed with the previous study that the level of lipid peroxidation was persistently increased from day 4 to day 14 after cerebral ischemia [Watson et al., 1996]. Increase in lipid peroxidation was resulted from the action of peroxynitrite formed from NO and superoxide anion (O$_2^•$) [Kumura et al., 1996]. The formation readily occurs as the rate constant of peroxynitrite is three times faster than the rate at which SOD scavenges O$_2^•$ [Gonzalez-Zulueta et al., 1998; Huie and Padmaja, 1993]. The direct relationship between the formation of peroxynitrite and the expression of iNOS was demonstrated by the study that an increased production of peroxynitrite occurred in the cortex at 22-46 hour after cerebral ischemia when iNOS was co-expressed [Suzuki et al., 2002]. Moreover, the product of lipid peroxidation further promoted the neuronal injury. 4-HNE could effectively inhibit the GPx activity by highly reacting with the reduced glutathione which was necessary for the activity of
GPx [Bosch-Morell et al., 1999; Romero et al., 1998]. Therefore, the H$_2$O$_2$ produced could not be effectively removed and thereby the lipid peroxidation was triggered [Hall et al., 1993]. In addition, in the brain tissues, activation of phospholipases which catalyses lipolysis and the peroxidation of polyunsaturated fatty acids causes increase in pool of arachidonic acid [İşlekel et al., 1999; Michowiz et al., 1990; Weglicki et al., 1984]. Metabolization of arachidonic acid with reperfusion will lead to overproduction of superoxide radicals and causes the elongation of lipid peroxidation of membranes [Imagawa et al., 1983]. As a result, the lipid peroxidation was greatly increased at the subacute stage of cerebral ischemia.

To sum up, the relationship of the generation of free radicals and the activities of corresponding antioxidative enzymes was shown in Figure 3.7. Firstly, the induction of cerebral ischemia-reperfusion activates the glutamate and its receptors, leading to the increase in intracellular Ca$^{2+}$ content, which in turns activates the NOS expression and activities (Stage 1 as indicated in Figure 3.7). Secondly, decreased activities of both SOD and GPx restricted the ability to reduce the superoxide anions from the mitochondrial leakage into non-reactive oxygen molecule (Stage 2 and 3). The accumulated superoxide anion readily reacted with the nitric oxide to form the peroxynitrite which was highly reactive (Stage 4). The hydroxyl radicals converted from peroxynitrite and hydrogen peroxide initiated the lipid peroxidation of membrane (Stage 5). It ended with the dramatic production of MDA and 4-HNE, and finally led to the cerebral infarction (Stage 6).
Figure 3.7 Events occurred after the cerebral ischemia-reperfusion in relation to the results obtained in the present study.
3.5 CONCLUSION

In conclusion, the cerebral ischemia-reperfusion induced in rat model is reproducible and consistent with other studies, in terms of an extensive production of NO, the transient upregulation of SOD and GPx activities, and the resulting exacerbation of lipid peroxidation.
CHAPTER 4

Antioxidative effect of electroacupuncture on reducing free radical-mediated toxicity induced by cerebral ischemia

4.1 INTRODUCTION

Nitric oxide (NO) plays a significant role in the neurotoxicity in brain tissues after cerebral ischemia-reperfusion. After transient cerebral ischemia, upregulation of NOS isoforms lead to the extensive production of NO, resulting in the neuronal cell death. Particularly, NO produced by upregulated nNOS and iNOS are detrimental to neurons. Detrimental effects of nNOS-produced NO, such as the increased infarct volume of the ischemic brain, could be reduced by the administration of nNOS inhibitors such as 7-nitroindazole, ARL17477 or s-methyl-isothioureido-L-norvaline [Nagafuji et al., 1995b; Yoshida et al., 1994; Zhang et al., 1996a]. Similarly, administration of aminoguanidine, an iNOS inhibitor, could reduce the damaging effects of NO [Iadecola et al., 1995a, 1996; Zhang et al., 1996b]. On the other hand, the NO produced by eNOS is cerebroprotective as it improves cerebral blood flow in situ within 2 hours of cerebral ischemia [Leker et al., 2001; Zhang et al., 1993]. The beneficial effects of endothelial NO cannot outweigh the neurotoxic potentials of neuronal and inducible NO produced [Iadecola, 1997]. As a whole, NO produced during cerebral ischemia is predominantly neurotoxic [Zhang et al., 1994]. As NO contributes to the pathogenesis of ischemic brain injury, the regulation of NO generation becomes a possible strategy as the treatment of stroke.
Despite of the inhibition of NO production, increasing the scavenging of free radicals, such as superoxide anion (O$_2$•$^-$) and hydrogen peroxide (H$_2$O$_2$), serves as another therapeutic strategy to stroke condition. Isoforms of mammalian SOD, copper/zinc SOD (CuZnSOD) and manganese SOD (MnSOD), are able to convert O$_2$•$^-$ to H$_2$O$_2$ [Michels et al., 1994; Okabe et al., 1998]. The generated H$_2$O$_2$ is subsequently removed into water and molecular oxygen by the glutathione peroxidase (GPx) [Crack et al., 2003]. However, the sudden burst of ROS after cerebral ischemia-reperfusion causes these antioxidant enzymes functionally inactive, resulting in triggered lipid peroxidation and exacerbated cerebral infarction. Recent studies demonstrate that increasing the functional levels of antioxidant enzymes can attenuate the ischemia-reperfusion injury. For instance, transgenic mice over-expressing either SOD or GPx showed significant reduction of infarct volume and edema formation [Chan et al., 1995; Fujimura et al., 2001; Kinouchi et al., 1998; Kokubo et al., 2002; Saito et al., 2001; Weisbrot-Lefkowitz et al., 1998]. An intravenous administration of polyethylene-conjugated SOD or GPx can also minimize the brain infarction [Francis et al., 1997; Liu et al., 1989; Tagaya et al., 1992]. Consistently, knockout mice lack of SOD or GPx showed an enhanced lipid peroxidation, leading to exacerbated infarction after transient focal cerebral ischemia [Chan et al., 1995; Fujimura et al., 2001; Kim et al., 2002]. Therefore, upregulating the activity of SOD and GPx suggests a protective role in cerebral ischemia.

Acupuncture and electro-acupuncture (EA) has been used to treat different types of diseases for many years in Asian countries, particularly in China, Korea, and Japan [Cheng, 1987]. In western countries, EA has been recently used for stroke rehabilitation. Clinical studies reported several beneficial outcomes, such as an
improved speech ability [Liu et al., 2000; Zhang and Ni, 1998; Zhao et al., 1998], improved locomotion [Liu et al., 1999; Tan, 1990; Zhang and Ni, 1998], and enhanced memory [Li and Shen, 1998]. Experimental studies found that EA attenuates the edema formation, lipid peroxidation as well as cerebral infarction [OuYang et al., 1999; Xu et al., 1996; Zhao et al., 2000]. Part of the mechanism for the attenuated neurotoxicity has been investigated in the animal stroke model only when a combination of acupoints is selected and stimulated [Kim et al., 2001; OuYang et al., 1999; Wei et al., 2000; Zhao et al., 2000]. Stimulation at a set of acupoints increases the complexity of finding the beneficial effects and the underlying mechanism on using each acupoint. In this study, therefore, each pair of bilateral acupoints is stimulated in an individual rat that can facilitate the understanding of resulting effects and outcomes. Furthermore, some acupoints have been reported to be effective only in certain kinds of diseases. None has postulated whether there is any direct relationship between the acupoint and the disease type. To explore the understanding on these two unclear areas, we selected and individually applied EA stimulation at a pair of bilateral acupoints, namely Neiguan (PC06), Fengchi (GB20), and Zusanli (ST36). These acupoints are commonly used for stroke rehabilitation [Di et al., 1993; Liu et al., 1999; Tan, 1990; Xing and Zhang, 1998; Zhang and Ni, 1998; Zhao et al., 1998] and treatment of cardiac diseases [Song et al., 1990; Song et al., 1993; Ye and Zhang, 1992]. The first part of this study is to determine the survival rate of EA-stimulated ischemic rats that is measured at 24 hours after induction of cerebral ischemia. The second part of study is to investigate if EA could regulate the NO production and the activities of antioxidative enzymes, and hence the overall outcome as indicated in the lipid peroxidation.
4.2 MATERIALS AND METHODS

4.2.1 Experimental design

Male Sprague-Dawley rats weighing 330-350 g were maintained with free access to food and water under a controlled environment (12 h dark/12 h light cycle, 20-22°C). Procedures were approved by the Animal Subject Ethics Sub-committee of the Hong Kong Polytechnic University. Seventy rats were randomly used for the determination of survival rates as described in Section 4.2.5. Another sixty-six rats were randomly assigned into eleven groups. The induction of cerebral ischemia and EA stimulation given in each group (n=6) were summarized in Table 4.1.

4.2.2 Induction of focal cerebral ischemia

Cerebral ischemia by occluding middle cerebral artery (MCAO) was induced according to the procedures previously described in Section 3.2.4. Briefly, after the ketamine-xylazine anesthesia, the temporalis muscle was separated in the plane of its fiber bundles to expose the zygoma and squamosal bone. A 5 mm x 5 mm burr hole was made to expose the right MCA, which was occluded for 1 h followed by reperfusion.

4.2.3 Application of electroacupuncture stimulaton

Being common, bilateral acupoints for stroke rehabilitation, Fengchi (GB20), Neiguan (PC06) and Zusanli (ST36) were selected and stimulated. In human, Fengchi are located on the posterior aspect of the neck, below the occipital bone, in the depression between the sternocleidomastoid muscles and trapezius muscles [Ellis et al., 1991]. In the rats, the points were located 3 mm away from the centre of a line joining two ears [Siu et al., 2004]. In human, Neiguan (PC06) is located on 2-cun above the
transverse crease of the wrist, between the tendons of the long palmar muscle (m. palmaris longus) and the radial flexor muscle (m. flexor carpi radialis) of the wrist [Ellis et al., 1991]. They are located 2 mm away from the transverse crease of the wrist of rats. In human, Zusanli is located 3” below the knee joint of the hind limb and 1” lateral to the crest of the tibia [Ellis et al., 1991]. In the rats, the points were located 5 mm below the knee joint of the hind limb and 2 mm lateral to the anterior tubercle of the tibia [Yun et al., 2002]. A single EA stimulation by EA stimulator (Model G6805-02, Smeif, Shanghai, China: Voltage 0.7V; Frequency 2Hz; Duration 0.5ms) was immediately applied for 30 min following induction of cerebral ischemia. In the second experiment of study, electrical stimulation was delivered to the groups receiving EA stimulation at GB20 (I1-GB and I4-GB) and EA stimulation at ST36 (I1-ST and I4-ST). No electrical stimulation was delivered to ischemic rats with Sham EA stimulation (I1-SGB, I4-SGB, I1-SST and I4-SST).

4.2.4 Determination of survival rates of EA-stimulated ischemic rats

Rats were assigned into four groups: (1) Ischemia (n=20); (2) Ischemia + EA-GB20 (n=20); (3) Ischemia + EA-PC06 (n=20); and (4) Ischemia + EA-ST36 (n=10). All rats were induced with transient focal cerebral ischemia. Two stainless steel needles were bilaterally inserted into points corresponding to Fengchi (GB20) in Ischemia + EA-GB20 group, to Neiguan (PC06) in the Ischemia + EA-PC06 group, and to Zusanli (ST36) in the Ischemia + EA-ST36 group. Except the Ischemia group, a single application of EA stimulation was immediately given at acupoints for 30 min. Number of rats survived in each group was counted at 24 hours of MCA occlusion. Survival rate of rats were expressed as the percentage derived from the number of survived rats to total number of rats in each group.
Table 4.1 Design of grouping of rats for the induction of transient cerebral ischemia and EA stimulation.

<table>
<thead>
<tr>
<th></th>
<th>Post-ischemia Day 1</th>
<th>Post-ischemia Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>I1</td>
</tr>
<tr>
<td>Cerebral ischemia</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sham EA-GB20</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sham EA-ST36</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>EA-GB20</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>EA-ST36</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Transient cerebral ischemia was induced by right middle cerebral artery occlusion (MCAO) for 1 hour followed by reperfusion. Rats designated for Sham EA-GB20 and Sham EA-ST36 treatments were inserted with acupuncture needles in Fengchi (GB20) and Zusanli (ST36), respectively, but without electrical stimulation. Rats assigned for EA-GB20 and EA-ST36 received electrical stimulation on bilateral acupuncture points, Fengchi and Zusanli, respectively. After MCAO, a single electrical stimulation was applied at voltage 0.7 V, frequency 2 Hz, duration 0.5 ms for 30 min. To identify the basal protein expression and activity, a group of normal, healthy rats was included and assigned as Normal group (N). Right brains of rats were obtained at either post-ischemia day 1 or day 4.
4.2.5 Chemicals and antibodies

Unless stated otherwise, all materials were of analytical grade and obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Primary antibodies of 200µg/ml probed against nNOS, iNOS, eNOS were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and used at 1:200 dilution. Polyclonal antibodies of CuZnSOD and MnSOD at 11 mg/ml were ordered from Calbiochem Co. (San Diego, CA, USA) and used at 1:200 dilution. Internal control using α-tubulin was polyclonal antibody obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibodies conjugated with horseradish peroxidase (HRP) directed against rabbit IgG and sheep IgG were obtained from Pierce Chemical Co. (Rockford, IL, USA) and Calbiochem Co. (San Diego, CA, USA), respectively, and used at a dilution of 1:1000. Protein concentration was determined by using protein assay as described by Bradford (1976) (Bio-Rad Laboratories, Hercules, CA, USA).

4.2.5 Assay of total NOS activity

NOS activity was determined by the total amount of NO produced from L-arginine by Griess reaction as previously described in Section 3.2.8. In short, one and four days after ischemia-reperfusion, the perfused right brains were harvested, weighed and homogenized mechanically. After centrifugation (10,000 x g, 30 min, 4°C), the supernatant was taken for 2-hour incubation with all cofactors. The incubated mixture was subsequently taken for the determination of NO according to the Griess reaction and the absorbance of end-products was measured at 540 nm. The total NOS activity was expressed as pmol nitrate produced per hour per mg protein.
4.2.6 Expression of NOS and SOD isoforms

Amount of NOS isoforms and SOD isoforms were detected from the western blots probed with specific primary antibodies. Procedures were in accordance with those previously described in Section 3.2.9. A 10% and 15% SDS-PAGE separating gel were prepared for the detection of NOS isoforms and SOD isoforms, respectively. In brief, homogenate protein (200 µg) resolved by gel electrophoresis were electroblotted to a nitrocellulose membrane at a constant voltage 25 V for 16 hr. After transfer, membranes were blocked with skimmed milk in 1X TBS-T and then probed with primary antibody raised against nNOS, eNOS iNOS, CuZnSOD or MnSOD overnight at 4ºC with gentle shaking. Subsequently, the probed blots were incubated with secondary antibodies with gentle shaking. The immunolabelled proteins (isoforms of NOS or SOD) were detected by reacting with chemiluminescent substrate (Pierce Chemical Co., Rockford, IL, USA). Chemiluminescence released was captured and quantified as pg NOS isoform or SOD isoform per mg protein. Selected blots were re-probed with α–tubulin as an internal control.

4.2.7 Assay of total SOD activity

This assay employed a commercially available assay kit specific for SOD in which the activity is determined by the rate of the superoxide-driven auto-oxidation of tetracyclic catechol in alkaline condition [Nebot et al., 1993] (Calbiochem Co., San Diego, CA, USA). The procedures were described in Section 3.2.10. The linearly increasing absorbance represented the rate of activity in the presence (Vs) and the absence (Vc) of SOD in samples and control blank, respectively. The total SOD activity was the relative activity derived from the ratio of Vs to Vc. Activity unit was defined as the activity that doubles the auto-oxidation rate of the control blank.
4.2.8 Assay of GPx activity

A coupled enzyme method was employed for determining GPx activity in which reduced NAPDH used for the regeneration of reduced glutathione (GSH) by glutathione reductase was produced by GPx activity (Calbiochem, San Diego, CA, USA) [Paglia and Valentine, 1967]. The assay procedures were described in Section 3.2.11. The linear portion of the decreasing absorbance at 340 nm represented the GPx activity. The activity unit was expressed as nmol NADPH oxidized per min per mg protein.

4.2.9 Lipid peroxidation assay

Malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE) was determined from the reaction with a chromogenic reagent to yield a stable products with maximum absorbance at 586 nm (Oxis International, Inc., Portland, OR, USA) [Erdelmeier et al., 1998]. Procedures were described in Section 3.2.12. The unit was defined as [MDA] (µM) per mg homogenate protein or [MDA + 4-HNE] (µM) per mg homogenate protein.

4.2.10 Statistical analysis

Data were expressed as mean ± standard deviation. Comparisons among different groups were made using multiple analyses of variance (ANOVA) followed by post-hoc protected least-significant different test. In all instances, \( P<0.05 \) was considered statistically significant.
4.3 RESULTS

4.3.1 Survival rate

Survival rate of rats subjected to transient focal cerebral ischemia with and without EA stimulation is shown in Table 4.2. Survival rate of ischemic rats without any remedy was 90%, while that of ischemic rats with EA stimulation at GB20 and ST36 were 95% and 100%, respectively. However, a dramatic decrease of survival rate (down to 15%) was found in ischemic rats with EA stimulation at PC06. This observation suggested that EA stimulation at PC06 was not good for treatment of cerebral ischemia.

4.3.2 Total NOS activity and expressions

Total NOS activity measured at day 1 and day 4 after cerebral ischemia was shown in Figure 4.1, and the abundance of NOS isoforms was obtained in Figure 4.2 to Figure 4.4. The Normal group had the total NOS activity at 290.43 ± 43.16 pmol nitrate produced per hr per mg protein. After reperfusion for 1 day, there was no induced total NOS activity in all the experimental groups compared to that in the Normal group. However, four days after ischemia-reperfusion, a nearly two-fold increase in total NOS activity was observed in the ischemic rats (I4 group) and the ischemic rats with sham EA stimulation (I4-SGB and I4-SST groups) (P<0.05). Two groups with EA stimulation at GB20 (I1-GB and I4-GB groups) were found to effectively reduce the total NOS activity to 257.78 ± 40.33 and 303.61 ± 15.07 pmol nitrate/hr/mg protein, respectively, which were comparable to the Normal group but significantly lower than two Control groups (I1 and I4 groups) (P<0.05). About one-fourth induced activity at post-ischemia day 4 was inhibited by EA stimulation at ST36 (I4-ST group) (P<0.05) but it was higher than those of the Normal group and I4-GB group (P<0.05).
Table 4.2 Survival rate of rats subjected to transient focal cerebral ischemia with or without electro-acupuncture at meridian point. Cerebral ischemia was induced by occlusion of middle cerebral artery (MCA) for 1 hour followed by reperfusion. Ischemic rats with EA stimulation were additionally subjected to 30-min EA at 2 Hz bilaterally at either Fengchi (GB20) or Neiguan (PC06) or Zusanli (ST36).

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Ischemia only</th>
<th>Ischemia + EA-GB20</th>
<th>Ischemia + EA-PC06</th>
<th>Ischemia + EA-ST36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats subjected to experimental conditions</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Number of survived rats after 24 hours</td>
<td>18</td>
<td>19</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Survival Rate</td>
<td>90%</td>
<td>95%</td>
<td>15%</td>
<td>100%</td>
</tr>
</tbody>
</table>
Figure 4.1 Total nitric oxide synthase (NOS) activity of right brains of normal and operated groups after 1-day or 4-day reperfusion following 1-hour MCAO and EA stimulation. Data represents mean ± S.D., n=6 in each group. Total NOS activity was determined by monitoring the total amount of NO produced from L-arginine by Griess reaction. Activity unit was defined as pmol nitrate produced per hr per mg protein. Symbol (a) represents the significant difference to the Normal group (P<0.05). On the same harvest day, symbols (b,c,e) denote the significant differences to the Ischemia group (P<0.05), and to the group with corresponding Sham EA stimulation (P<0.05), between experimental groups with EA stimulation at two acupuncture points (P<0.05), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups (P<0.05).
Abundance of NOS isoforms was almost consistent with that in the total NOS activity. In the Normal group, the amount of expressed isoforms were 222.29 ± 7.03 pg nNOS per mg protein, 75.66 ± 9.76 pg iNOS per mg protein and 119.34 ± 4.97 pg eNOS per mg protein. In all the experimental groups at day 1 after ischemia-reperfusion, both nNOS and iNOS expressions were not altered, while eNOS expression was suppressed (P<0.05). At day 4 after ischemia-reperfusion, the expression of each isoform in the ischemic rats (I4 group) and the ischemic rats with sham EA stimulation (I4-SGB and I4-SST groups) was greatly up-regulated by two times (P<0.05). In parallel to the total NOS activity, EA stimulation at GB20 was more effective in suppressing the nNOS and iNOS. Their expressions were reduced to 138.98 ± 24.15 pg nNOS per mg protein and 26.45 ± 2.74 pg iNOS per mg protein at post-ischemia day 1, that were significantly different from those of the Normal group and I1 group (P<0.05). Likewise, both nNOS and iNOS expressions in the I4-GB group were significantly decreased by 55% in comparison to the I4 group and I4-SGB group (P<0.05). However, the EA stimulation at ST36 was only able to inhibit the expression of nNOS and iNOS at post-ischemia day 4, in which about 40% of inhibition was observed in comparison to the I4 group (P<0.05). Unlike the regulation of nNOS and iNOS expression, the eNOS expression was only transiently induced in the I4-GB group and I4-ST group and their expression was restored to half of the eNOS amount detected in the Normal group. However, the EA stimulation suppressed the upregulation of eNOS after 4 days of ischemia-reperfusion (P<0.05). Only EA stimulation at ST36 could produce the eNOS expression that was significantly higher than that in the Normal group (P<0.05).
Figure 4.2 Abundance of neuronal nitric oxide synthase (nNOS) of right brains of normal and operated groups after 1-day or 4-day reperfusion following 1-hour MCAO and EA stimulation. Data represents mean ± S.D., n=6 in each group. The unit was expressed as pg nNOS per mg homogenate. Symbol (a) represents the significant difference to the Normal group ($P<0.05$). On the same harvest day, symbols (b,c,e) denote the significant differences to the Ischemia group ($P<0.05$), and to the group with corresponding Sham EA stimulation ($P<0.05$), between experimental groups with EA stimulation at two acupuncture points ($P<0.05$), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups ($P<0.05$).
Figure 4.3 Abundance of inducible nitric oxide synthase (iNOS) of right brains of normal and operated groups after 1-day or 4-day reperfusion following 1-hour MCAO and EA stimulation. Data represents mean ± S.D., n=6 in each group. The unit was expressed as pg iNOS per mg homogenate. Symbol (a) represents the significant difference to the Normal group (P<0.05). On the same harvest day, symbols (b,c,e) denote the significant differences to the Ischemia group (P<0.05), and to the group with corresponding Sham EA stimulation (P<0.05), between experimental groups with EA stimulation at two acupuncture points (P<0.05), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups (P<0.05).
Figure 4.4 Abundance of endothelial nitric oxide synthase (eNOS) of right brains of normal and operated groups after 1-day or 4-day reperfusion following 1-hour MCAO and EA stimulation. Data represents mean ± S.D., n=6 in each group. The unit was expressed as pg eNOS per mg homogenate. Symbol (a) represents the significant difference to the Normal group ($P<0.05$). On the same harvest day, symbols (b,c,e) denote the significant differences to the Ischemia group ($P<0.05$), and to the group with corresponding Sham EA stimulation ($P<0.05$), between experimental groups with EA stimulation at two acupuncture points ($P<0.05$), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups ($P<0.05$).
4.3.3 Total SOD activity and expressions

Results of total SOD activity and expression of isoforms obtained were shown in Figure 4.5 to Figure 4.7. In Figure 4.5, the total SOD activity of the Normal group was found at 0.924 ± 0.11. After induction of transient cerebral ischemia for 1 day, the total SOD activity in the I1 group was significantly increased to 1.2 ± 0.2, which was significantly higher than those of the Normal group (P<0.05). Total SOD activities were further increased by 30% and 24% in ischemic rats with EA stimulation at GB20 (I1-GB group) (P<0.05) and EA stimulation at ST36 (I1-ST group) (P<0.05), respectively. However, at post-ischemia day 4, the total SOD activity in the I4 group was restored to normal. Only EA stimulation at ST36 was found to maintain total SOD activity at 1.39 ± 0.08, which was significantly to other corresponding experimental groups (P<0.05).

Referring to Figure 4.6 and Figure 4.7, the CuZnSOD and MnSOD was transiently upregulated in ischemic rats (I1 group) after 1 day of ischemia-reperfusion (P<0.05). At post-ischemia day 4, CuZnSOD expression was then reduced to the level that was significantly lower than that of the Normal group (P<0.05). EA stimulation at GB20 was only able to further stimulate the expression of CuZnSOD and MnSOD by 10% at post-ischemia day 1. On the other hand, EA stimulation at ST36 could maintain their expression at 2.94 ± 0.31 pg CuZnSOD per mg protein and 5.79 ± 0.26 pg MnSOD per mg protein at post-ischemia day 4, that was significant to the corresponding ischemic rats (I4 group) and ischemic rats with Sham EA stimulation (I4-SST group) (P<0.05). None of groups with the Sham EA stimulation (I1-SGB, I1-SST, I4-SGB, I4-SST groups) could increase the total SOD activity or their expression, compared to the corresponding ischemic rats.
Figure 4.5 Total superoxide dimutase (SOD) activity of right brains of normal and operated groups after 1-day or 4-day reperfusion following 1-hour MCAO and EA stimulation. Data represents mean ± S.D., n=6 in each group. Total SOD activity was determined by quantifying the reaction products in superoxide-driven auto-oxidation of tetracyclic catechol. One activity unit was defined as an activity doubling the auto-oxidation rate of tetracyclic catechol. Symbol (a) represents the significant difference to the Normal group (\(P<0.05\)). On the same harvest day, symbols (b,c,e) denote the significant differences to the Ischemia group (\(P<0.05\)), and to the group with corresponding Sham EA stimulation (\(P<0.05\)), between experimental groups with EA stimulation at two acupuncture points (\(P<0.05\)), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups (\(P<0.05\)).
Figure 4.6 (A) Copper/Zinc superoxide dismutase (CuZnSOD) expression pattern of normal and operated brains harvested after transient focal ischemia at post-ischemia day 1 and day 4; (B) the abundance of them was quantified as pg CuZnSOD per mg protein. The data represents mean ± S.D., n=6 in each group. An internal control, α-tubulin, was selectively probed. Symbol (a) represents the significant difference to the Normal group ($P<0.05$). On the same harvest day, symbols (b,c,e) denote the significant differences to the Ischemia group ($P<0.05$), and to the group with corresponding Sham EA stimulation ($P<0.05$), between experimental groups with EA stimulation at two acupuncture points ($P<0.05$), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups ($P<0.05$).
Figure 4.7 (A) Manganese superoxide dismutase (MnSOD) expression pattern of normal and operated brains harvested after transient focal ischemia at post-ischemia day 1 and day 4; (B) the abundance of them was quantified as pg MnSOD per mg protein. The data represents mean ± S.D., n=6 in each group. An internal control, α-tubulin, was selectively probed. Symbol (a) represents the significant difference to the Normal group (P<0.05). On the same harvest day, symbols (b,c) denote the significant differences to the Ischemia group (P<0.05), and to the group with corresponding Sham EA stimulation (P<0.05), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups (P<0.05).
Figure 4.8 Glutathione peroxidase (GPx) activity of right brains of normal and operated groups after 1-day or 4-day reperfusion following 1-hour MCAO and EA stimulation. Data represents mean ± S.D., n=6 in each group. The activity unit was expressed as nmol NADPH oxidized per min per mg protein. Symbol (a) represents the significant difference to the Normal group \((P<0.05)\). On the same harvest day, symbols (b,c,e) denote the significant differences to the Ischemia group \((P<0.05)\), and to the group with corresponding Sham EA stimulation \((P<0.05)\), between experimental groups with EA stimulation at two acupuncture points \((P<0.05)\), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups \((P<0.05)\).
Figure 4.9 Levels of lipid peroxidation products, (A) only malondialdehyde (MDA) and (b) MDA + 4-HNE (4-hydroxy-2(E)-nonenal), measured in the normal and operated rats. LPX unit was defined as [MDA] (µM) or [MDA + 4-HNE] (µM) per mg protein. Symbol (a) represents the significant difference to the Normal group ($P<0.05$). On the same harvest day, symbols (b,c,e) denote the significant differences to the Ischemia group ($P<0.05$), and to the group with corresponding Sham EA stimulation ($P<0.05$), between experimental groups with EA stimulation at two acupuncture points ($P<0.05$), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups ($P<0.05$).
4.3.4 GPx activity

GPx activities was measured in normal and operated rats after 1 day and 4 days of cerebral ischemia-reperfusion (Figure 4.8). The Normal group exhibited a GPx activity at 281.86 ± 7.39 nmol NADPH oxidized per min per mg protein. After the induction of cerebral ischemia for 1 day, the GPx activity of the I1 group was significantly increased to 302.86 ± 5.33 nmol NADPH oxidized/min/mg protein (P<0.05). Such a transient increase was declined to the normal level at post-ischemia day 4. EA stimulation at GB20 was only able to further induce the GPx activity at post-ischemia day 1, in comparison to the ischemic rats (I1 group) and ischemic rats with Sham EA stimulation at GB20 (I1-SGB group) (P<0.05). Likewise, compared to the corresponding ischemic rats, EA stimulation at ST36 could significantly increase the GPx activities at both post-ischemia day 1 and 4 (P<0.05).

4.3.5 Lipid peroxidation

Levels of lipid peroxidation, in terms of MDA and 4-HNE, measured were shown in Figure 4.9. There were 26.19 ± 4.2 µM MDA per mg protein and 86.62 ± 3.85 µM MDA+4-HNE per mg protein detected in the Normal group. Upon the induction of cerebral ischemia, MDA and MDA+4-HNE were progressively increased from day 1 to day 4 (P<0.05). As observed in the ischemic rats with Sham EA stimulation (I1-SGB, I1-SST, I4-SGB, I4-SST groups), there was no altered levels of lipid peroxidation found at post-ischemia day 1 and 4. In comparison to the I1 group, the MDA and MDA+4-HNE levels in I1-GB group were reduced by 10% (P<0.05). However, no reduction of lipid peroxidation was observed between I4 group and I4-GB group. EA stimulation at ST36 could only decrease the levels of lipid peroxidation to 59.14 ± 9.18 µM MDA per mg protein and 135.62 ± 10.48 µM
MDA+4-HNE per mg protein after 4 days of cerebral ischemia, that were significantly lowered than those in other experimental groups (P<0.05).

4.4 DISCUSSION

Selection of acupoints is believed to be critical in providing therapeutic effects on a disease but few studies have investigated if it is disease-specific. This study has therefore explored such a relationship by monitoring the survival rate of ischemic rats with an individual, bilateral EA stimulation at Fengchi (GB20), Neiguan (PC06) or Zusanli (ST36). GB20 is commonly used in brain diseases [Jiang et al., 1982; Tan, 1990; Zhao et al., 1998; Zou and Wang, 1990], PC06 in heart-related diseases, and ST36 in brain and gastrointestinal diseases [Ji et al., 1987; Research School of Chinese Medicine, 1979; Ruan, 1980; Xu and Cui, 1998; Zhang et al., 2001]. Previous studies found that EA stimulation at Neiguan could raise blood pressure, protect cardiac pump function and reduce blood adhesion, so as to ameliorate the acute ischemic myocardial injury and hemorrhage shock [Cao et al., 1998; Chen et al., 1982; Chen et al., 1986; Lin et al., 1997; Yang and Wang, 1980; Yang et al., 1985; Zhao et al., 1985]. Conversely, our results showed that most of the ischemic rats were dead after cerebral ischemia and EA stimulation at PC06. Such a low survival rate was not observed in other groups. Failure to promote the survival of rats was probably because the time to deliver EA stimulation at PC06 was too early after reperfusion. Previous studies had reported that EA stimulation at PC06 could reduce cerebral infarction when it was applied days from the onset of stroke [Du and Di, 2000; Li et al., 1995; Ma et al., 2001; Wu, 2001; Wu and Cao, 1998; Xing and Zhang, 1998; Wang and Wang, 2001]. The strengthened ventricular muscle contraction
triggered by EA stimulation at PC06 increased the cerebral blood flow that may worsen the damage induced by ischemia-reperfusion [Huang et al., 1998; Yang et al., 1985]. On the other hand, it was not surprisingly to find that EA stimulation at GB20 or ST36 showed no effect on the survival of ischemic rats because of their proper regulation of the blood flow and circulation of ischemic brains [Shi et al., 1997; Tan, 1990; Zhou et al., 1993; Wang and Wang, 1995]. These observations may suggest that the some acupoints are specific for a particular disease.

We then investigated whether EA stimulation at GB20 or ST36 can regulate the NO production and modulate the scavenging activities of antioxidative enzymes in brain tissues. Both NOS activity and expressions of NOS were found to be inhibited by EA stimulation at GB20 or ST36 and the extent of inhibition was greater when stimulating at GB20. Reduction of NO production had been reported by Zhao and colleagues (Zhao et al., 2000). Their study showed that NO production was attenuated at the rat striatum during two hours of transient cerebral ischemia, but NO production was measured afterwards. Our results additionally showed that NO production at the subacute stage of ischemia could also be regulated by EA stimulation. Since NO production was progressive and time-dependent, further regulation at the subacute stage may provide greater protection to brain tissues. Especially the decreased nNOS and iNOS protein could reduce the cerebral infarct volume [Fassbender et al., 2000; Nagafuji et al., 1995b; Yoshida et al., 1994; Zhang et al., 1996]. The decreased NOS activity may be possibly due to the suppressed release of excitatory amino acids that triggered the influx of extracellular calcium [Zhao et al., 1997]. It hence inhibited the calcium-dependent nNOS activity that contributes to the total NOS activity. Also, reduced iNOS expression was likely
associated with the down-regulated mRNA and protein expression of interleukin-1 (IL-1) after EA stimulation [Xu et al., 2002; Zhang et al., 1994] and the down-regulation of nNOS through the transcription factor nuclear factor kappaB (NFκB) [Togashi et al., 1997]. Unlike nNOS and iNOS, eNOS was expressed more at post-ischemia day 1 but was suppressed at post-ischemia day 4 after EA stimulation at GB20 or ST36. But eNOS expression level of ischemic rats with EA stimulation at ST36 was slightly higher than that of the Normal group. Previous studies reported that EA stimulation could increase the amount of basic fibroblast growth factor (bFGF) in striatum and cortex one day after cerebral ischemia [Ou et al., 2001], which in turns enhanced the eNOS expression [Hossmann, 1994; Leker et al., 2001; Nagafuji et al., 1995a]. Endothelial NO produced is able to dilate blood vessels of injury sites to compensate for the reduced cerebral blood flow during ischemia, so as to properly maintain the intact blood-brain barrier [Leker et al., 2001; Komatsu et al., 1999; Zhang et al., 1993]. However, the reduced eNOS measured in this study may be associated with the decreased expression of CuZnSOD which could induce the eNOS expression and protect it from the protein nitration induced by NO that was vigorously produced by nNOS and iNOS [Brennan et al., 2002; Brennan et al., 2003; Kim et al., 2001].

EA stimulation at GB20 or ST36 could potentiate the activities of antioxidant enzymes (both SOD and GPx). The induced activities were only observed in day 1 when EA stimulation was applied to GB20, but were observed in both day 1 and day 4 when EA stimulation was applied to ST36. It seems that EA stimulation could produce a longer period of activation of antioxidant enzymes. In other traumatic injuries or neuronal diseases treated with EA stimulation, upregulated SOD activities
with decreased MDA content were observed [Lai et al., 2000; Wu et al., 1999]. The upregulated expressions of SOD could probably produce several beneficial effects to brain tissues after transient focal ischemia, such as the enhanced recovery of ischemic cellular edema [Kokubo et al., 2002], the attenuation of the destruction of blood brain barrier [Francis et al., 1997], the reduction of DNA fragmentation as a result of apoptosis induced [Martz et al., 2001]. However, they had not investigated the involvement of GPx on decreasing H$_2$O$_2$ and lipid peroxidation. Low expression and activity of catalase in brain minimized its ability in removing H$_2$O$_2$ [de Haan et al., 1998; Huang and Philbert, 1995; Yasmineh and Theologides, 1993]. In contrast to catalase, GPx had a higher tolerance to ischemic insults or hypoxia [Crack et al., 2003; Michels et al., 1994]. Thus the removal of H$_2$O$_2$ or phospholipid hydroperoxide was mainly dependent on the GPx activity. If excess SOD converted O$_2^-$ to H$_2$O$_2$ without a concomitant increase of GPx, an accumulated H$_2$O$_2$ would also lead to greater lipid peroxidation [Crack et al., 2003; Trépanier et al., 1996; Weisbrot-Lefkowitz et al., 1998]. Therefore, in using EA stimulation at ST36, the amount of lipid peroxidation products, MDA and 4-HNE, were both significantly diminished, which was consistent with the concomitantly increasing activities of SOD and GPx. EA stimulation at ST36 may likely provide a better and appropriate balance between SOD and GPx activities that may be in response to the accumulation of lipid peroxides or may reflect the healing process of ischemic necrosis triggered by EA stimulation [Takizawa et al., 1994]

The application of electrical stimulation was essential to produce the aforementioned neuroprotective effect. A single application of EA on Fengchi, not manual acupuncture, was able to regulate the cerebral blood flow and pressure [Yuan et al.,
In our experiment, neither enzymatic activities nor lipid peroxiation was modified in the ischemia rats with Sham EA stimulation where no electrical stimulation was given to the inserted acupuncture needles. These observations may suggest that EA could produce some additional beneficial effects than manual acupuncture.

To sum up, according to the events occurred after ischemia-reperfusion illustrated in Figure 4.10 (A) (modified from Figure 3.7 in Chapter 3), the beneficial effect of EA stimulation, particularly EA stimulation at ST36, were presented in a schematic diagram in Figure 4.10 (B). Firstly, after EA stimulation, the ischemia-reperfusion-induced activity and expression of NOS were suppressed (Stage 1 as indicated in Figure 4.10(B)). Simultaneously, the concomitantly increased activities of SOD and GPx accelerated the removal of superoxide anions generated by ischemia-reperfusion (Stage 2 and 3). It resulted in the production of the non-reactive oxygen and water molecules that would not react with the nitric oxide. The diminished production of nitric oxide and reduced availability of superoxide anion prevented the formation of peroxynitrite (Stage 4). In addition, the increasing activities of GPx avoided the formation of hydroxyl radical by effectively removing the hydrogen peroxide (Stage 5). As a whole, the lipid peroxidation process was suppressed as indicated by the reduced amount of MDA and 4-HNE (Stage 6).
Figure 4.10 (A) Events occurred after cerebral ischemia-reperfusion, and (B) events occurred after the cerebral ischemia-reperfusion followed by EA stimulation.
4.5 CONCLUSION

To conclude, this study has illustrated that the effect exerted by EA stimulation at some acupoints are disease-specific. EA stimulation at GB20 or ST36 can inhibit the NO production and potentiate the antioxidative enzymes activities, resulting in the attenuation of lipid peroxidation. It is found that EA stimulation at ST36 could produce a longer cellular defence to neurotoxicity at the subacute stage of transient focal cerebral ischemia.
CHAPTER 5

Electroacupuncture potentiates the thioredoxin expression in ischemia-reperfused rat brains

5.1 INTRODUCTION

Protein thiols and disulphides always fill essential structural, regulatory and catalytic roles in proteins. Some proteins require the protein thiols for their enzymatic activities, while some require the intact protein disulphides for their catalytic activities. Under a normal physiological condition, a relatively stable intracellular thiol-redox environment is always maintained [Ferret et al., 2000]. Thus the biological activities of enzymes and proteins are not easily modulated by the oxidation of protein thiols or the reduction of protein disulphides. However, after cerebral ischemia-reperfusion, a huge production of reactive oxygen species (ROS) has altered this properly balanced thiol-redox environment. It usually leads to the oxidation of protein thiols of some enzymes that eventually lose their normal biological activities. The unnecessary oxidation of protein thiols can be reverted by the thioredoxin system. The thioredoxin system consists of thioredoxin (Trx), thioredoxin reductase (TR) and NADPH [Luthman and Holmgren, 1982]. It promotes the reduction of ROS-induced protein disulphides by NADPH through a combination of reactions as described in Equation 5.1 and 5.2.

\[
\text{Thioredoxin-S}_2 + \text{NADPH} + \text{H}^+ \xrightarrow{\text{thioredoxin reductase}} \text{Thioredoxin-(SH)}_2 + \text{NADP}^+ \quad \text{Equation (5.1)}
\]

\[
\text{Thioredoxin-(SH)}_2 + \text{Protein-S}_2 \xrightarrow{\text{}} \text{Thioredoxin-S}_2 + \text{Protein-(SH)}_2 \quad \text{Equation (5.2)}
\]
Thioredoxin system has been regarded as an antioxidant system that can protect the cells from ROS-mediated toxicity. TR can directly reduce the S-nitroglutathione (GSNO), which is formed after glutathione readily reacts with activated nitric oxide intermediates [Burton, 1985; Gaston et al., 1993]. In cooperation with NADPH and TR, Trx can efficiently scavenge reactive oxygen intermediates, such as hydrogen peroxide and hydroxyl radicals [Arnér and Holmgren, 2000; Gromer et al., 1999; Mustacich and Powis, 2000; Nakamura et al., 1994; Poole et al., 2000; Takagi et al., 1998]. Therefore, the thioredoxin system can participate in the detoxification of lipid hydroperoxides [Björnstedt et al., 1994; Björnstedt et al., 1995]. In vitro studies also reported that Trx was able to reduce active oxygen species induced by xanthine-xanthine oxidase in the pulmonary ischemia-reperfusion injury [Mitsui et al., 1992]. Apart from its ROS-scavenging ability, Trx may play a role in keeping enzyme active under the oxidative stress. A typical example is the glycolytic enzyme, glyceraldehydes-3-phosphate dehydrogenase, in which Trx reactivates the thiol groups being inactivated by ROS and restores its activity [Fernando et al., 1992; Nakamura et al., 1994]. In addition, Trx has also been implicated in the regulation of cellular signaling pathways. For example, it reduces the redox factor (Ref-1) that controls the binding of Fos/Jun to activator protein-1 (AP-1) which in turn mediates transcriptional control of glutathione S-transferase [Abate et al., 1990; Hirota et al., 1997; Peunova and Enikolpov, 1993; Schenk et al., 1994]. It also acts as a potent growth promoting factor for the HIV-infected T-lymphocytes [Yodoi and Tursz, 1991]. Moreover, it can inhibit the apoptotic cascade by inactivating the apoptosis signal-regulating kinase (ASK-1) that promotes the mitogen-activated protein (MAP) kinase/p38 pathway [Saitoh et al., 1998]. Other examples of antiapoptotic actions of
Trx include the suppression of cytochrome c release, the induction of Bcl-2 expression, and the inhibition of caspase-3 and -9 [Andoh et al., 2002]

The thioredoxin system is widespread in different tissues [Lippoldt et al., 1995]. High levels of Trx and TR are present in nervous tissues, such as nerve cells and their axons [Rozell et al., 1985; Stemme et al., 1985]. However, only Trx, not TR, has been identified as a stress-inducible protein. Its expression can be upregulated by various types of stress, such as the viral infection, the exposure to ultraviolet light, the x-ray irradiation, and oxidative stress induced by hydrogen peroxide [Nakamura et al., 1994; Ohira et al., 1994; Sachi et al., 1995]. Upregulated expression was also reported after head trauma [Lippoldt et al., 1995], motor nerve injury [Mansur et al., 1998], and ischemia-reperfusion injury [Shibuki et al., 1998; Takagi et al., 1998]. These overexpressions may have multifunctional roles in protecting cells against ROS-mediated toxicity.

Electro-acupuncture (EA) has produced a variety of beneficial effects to stroke patients. Some improved clinical outcomes, such as reduced speech retardation [Liu et al., 2000; Zhang and Ni, 1998; Zhao et al., 1998], improved locomotion [Liu et al., 1999; Tan, 1990; Zhang and Ni, 1998], and enhanced memory [Li and Shen, 1998], have been reported. On the other hand, EA has reduced the neurotoxicity of the experimentally induced cerebral ischemia. For instances, EA attenuates the edema formation, lipid peroxidation and cerebral infarction as well [OuYang et al., 1999; Xu et al., 1996; Zhao et al., 2000]. One possible mechanism of this attenuation was inhibiting the production of nitric oxide that initiated the cascade of neuronal damage [Xu et al., 1996; Zhao et al., 2000]. Another possible mechanism is reducing the lipid
peroxidation by promoting the scavenging of reactive oxygen species, such as superoxide anion and hydrogen peroxide [Siu et al., 2004]. However, little has been investigated on the implication of EA on regulating the thiol-redox environment, so as to inhibit the redox-mediated oxidation of protein disulphides. In the present study, we thus aimed at investigating the possibility of EA on potentiating the activity of thioredoxin system, so as to maintain a thiol-redox environment after transient focal cerebral ischemia.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

Male Sprague-Dawley rats weighing 330-350 g were maintained with free access to food and water under a controlled environment (12 h dark/12 h light cycle, 20-22°C). A total of sixty-six rats were randomly assigned into eleven groups (each group n=6) according to Table 1 as previously described in Section 4.2.1.

5.2.2 Induction of focal cerebral ischemia

Cerebral ischemia by occluding middle cerebral artery (MCAO) was induced according to the procedures as previously described in Section 3.2.4. Rats were anesthetized with an intraperitoneal injection of ketamine and xylazine (Alfasan, Holland). Briefly the temporalis muscle was separated in the plane of its fiber bundles to expose the zygoma and squamosal bone where a hole was made. The exposed right MCA was occluded for 1 h followed by reperfusion for 1 and 4 days.
5.2.3 Application of electroacupuncture stimulation

Procedures of EA stimulation were in accordance with those previously described in Section 4.2.3. A single EA stimulation was applied for 30 min following MCAO. Two acupoints commonly used for treating stroke patients, Fengchi (GB20) and Zusanli (ST36), were selected. For the rats receiving Sham EA stimulation at GB20 or EA stimulation at GB20, two stainless steel needles were bilaterally inserted into points corresponding to GB20. For the rats receiving Sham EA stimulation at ST36 or EA stimulation at ST36, two needles were bilaterally inserted into points representing ST36. Electrical stimulation was delivered with EA stimulator (Model G6805-02, Smeif, Shanghai, China) at voltage 0.7 V, frequency 2 Hz, duration 0.5 ms, to the groups receiving EA stimulation at GB20 (I1-GB and I4-GB) and EA stimulation at ST36 (I1-ST and I4-ST). No electrical stimulation was delivered to ischemic rats with Sham EA stimulation (I1-SGB, I4-SGB, I1-SST and I4-SST).

5.2.4 Preparation of brain homogenate

After 1 day and 4 days of reperfusion, right hemispheres of brains of each group were removed, weighed, homogenized in 2 volumes of in ice-cold 20 mM Tris-HCl buffer (pH 7.4) added with protease inhibitor cocktail (P8340, Sigma, Saint Louis, MO, USA) and centrifuged for 30 min at 10,000 x g. The supernatant was collected for biochemical analysis. Total protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

5.2.5 TR activity on DTNB reduction

Mammalian TR has broader substrate specificity. In the presence of NADPH, it can directly reduce small, disulphide-containing molecules, such as 5,5’-dithiobis(2-
nitrobenzoic acid) (DTNB). This assay was based on its activity to catalyze the NADPH-dependent reduction of disulphide bonds of DTNB molecules [Luthman and Holmgren, 1982]. The reaction produced the yellow-coloured monomers, 2-nitro-5-thiobenzoate (TNB), with the maximum absorbance at 412 nm. Brain homogenate (100 µl) was added with 0.9 ml assay mixture (100 mM K₂HPO₄·3H₂O, pH 7.13, 10 mM EDTA, 5 mM DTNB, 0.2 mM NADPH) into a cuvette. The reaction was subsequently and constantly monitored at 412 nm for 2 min by spectrophotometer (Model U-2000, Hitachi, Japan). As 1 mole of NADPH yielded 2 moles of TNB, a correction factor of 0.0184 derived from 0.5/(13.6 x 2) was made. Hence TR activity was calculated using \( \Delta A_{412}/ \text{min} \times 0.0184 \). The activity unit was defined as µmoles NADPH oxidized per minute per mg protein.

5.2.6 TR activity on reduction of immunoglobulin molecules

In addition, with the aid of Trx and NADPH, TR can reduce the protein disulphide bridges in larger molecules, such as immunoglobulin G (IgG) employed in this study. This method relied on its reducing action on the inter-heavy-light chain and inter-heavy chain disulphides of IgG [Magnusson et al., 1997]. The functional activity level of brain TR was measured using commercially available Trx obtained from *Spirulina* species (T3658, Sigma, Saint Louis, MO, USA). A positive control was included using the purified TR obtained from rat liver (Activity level was 14 µmol NADPH oxidized per min per mg protein). In 50 µl incubation mixture, 10 µl brain homogenate was incubated with 200 ng rabbit intact IgG, 5 µM *Spirulina* Trx and 50 µM NADPH in 20 mM Tris-HCl (pH 7.4) for 30 min at 37ºC with gentle shaking. The incubated reaction mixture was boiled with 50 µl 1X non-reducing SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% bromophenol...
blue) for 10 min. The boiled reaction mixture was then resolved by SDS-PAGE using 10% separating gel and 4% stacking gel in Mini-Protean II Dual Slab Cell (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were electro-blotted at 25 V onto a 0.22 µm nitrocellulose membrane overnight. After probing with anti-rabbit IgG horseradish peroxidase (HRP)-conjugated (Pierce Chemical Co., Rockford, IL, USA; 1:8,000) for 1 h, the intact and cleaved IgG molecules were detected by reacting with Supersignal® West Pico Chemiluminescent Substrate (Pierce Chemical Co.). Chemiluminescence released was captured by a UVP-Chemi System (UVP, Inc., Upland, CA, USA). The amount of IgG was quantified by the Lab-Works™ Image Acquisition and Analysis Software (UVP, Inc., Upland, CA, USA). TR activity was determined as percentage reduction of intact IgG per mg homogenate protein.

5.2.7 Trx expression

The abundance of brain Trx was quantified by western blotting. Briefly, 200 µg brain homogenate was resolved by SDS-PAGE (12% separating gel and 4% stacking gel) and electro-blotted onto a nitrocellulose membrane. The membrane was first probed with mouse IgG raised against rat Trx (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 1:200), followed by probing with anti-mouse IgG HRP-conjugated antibodies for 2 hours with gentle shaking. Detection and quantification of brain Trx was in accordance with the aforementioned procedures described in Section 3.2.9. The expression unit was defined as pg Trx per mg homogenate protein. Selected blots were re-probed with rabbit IgG against rat α-tubulin as an internal control.
5.2.8 Statistical analysis
Data were expressed as mean ± standard deviation. Multiple analyses of variance (ANOVA) followed by post-hoc protected least-significant different test was used for comparison. In all instances, \( P < 0.05 \) was considered statistically significant.

5.3 RESULTS
5.3.1 TR activity on DTNB reduction
In the presence of NADPH, TR activity was determined from the reduction of DTNB by brain homogenate obtained 1 day or 4 day after transient focal ischemia (Figure 5.1). TR activity measured in the Normal group (4.78 ± 1.09 nmol NADPH oxidized per min per mg protein) was comparable to those in the Ischemia Day 1 group (5.233 ± 0.63) and Ischemia Day 4 group (5.185 ± 1.08). At either post-ischemia day 1 or day 4, statistical analysis revealed that there was no significant difference observed among all the experimental groups. These observations suggested that TR activity on reducing small disulphide-containing molecules was not regulated by the induction of cerebral ischemia or EA stimulation.

5.3.2 TR activity on reduction of IgG molecules
In the presence of both NADPH and Trx, TR activity on reducing protein disulphide bridges in IgG molecules was measured. Reduction pattern of the intact IgG molecules was obtained (Figure 5.2A) and the percentage of IgG reduction was derived after quantifying the bands corresponding to the intact IgG molecules and the cleaved IgG molecules (Figure 5.2B). The percentage of IgG reduction by TR obtained from the Normal group was 7.00 ± 0.78 %.
Figure 5.1. Thioredoxin reductase (TR) activity of right brains of normal and operated groups after 1-day or 4-day reperfusion following 1-hour MCAO. Data represents mean ± S.D., n=6 in each group. TR activity was determined from an initial increase of DTNB reduction as monitored at 412 nm in 100 mM Tris-HCl (pH 7.0), 5 mM DTNB, 1 mM NADPH. Activity unit was defined as nmoles of NADPH oxidized per min per mg protein. Statistical analysis revealed that no significant difference was observed.
### Figure 5.2. Reduction of IgG protein disulphide by brain TR of experimental groups after transient focal cerebral ischemia for 1 day or 4 days.

Brain homogenate was incubated with 200 ng rabbit IgG, 5 μM *Spirulina* Trx and 50 μM NADPH in 20 mM Tris-HCl (pH 7.4). After incubation at 37°C for 30 min, all the reaction mixtures were boiled and subjected to SDS-PAGE. Proteins in the gel were transferred onto a nitrocellulose membrane. It was then blocked with 5% skimmed milk, followed by conjugating with polyclonal anti-rabbit IgG HRP-conjugate. Intact and cleaved IgG molecules were detected by the means where the released chemiluminescence was captured and quantified. TR activity was determined as percentage IgG reduction per mg homogenate protein. Data represents mean ± S.D., n=6 in each group. Symbols (a, e) represent the significant difference to the Normal group (*P*<0.05), and between experimental groups with EA stimulation at two acupuncture points harvested at the same day (*P*<0.05), respectively.
Figure 5.3. (A) Trx expression pattern of normal and operated brains harvested after transient focal ischemia at post-ischemia day 1 and day 4; (B) the abundance of them was quantified as pg Trx per mg protein. The data represents mean ± S.D., n=6 in each group. An internal control, α-tubulin, was selectively probed. Symbol (a) represents the significant difference to the Normal group (P<0.05). On the same harvest day, symbols (b,c) denote the significant differences to the Ischemia group (P<0.05), and to the group with corresponding Sham EA stimulation (P<0.05), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups (P<0.05).
At post-ischemia day 1, all the experimental groups maintained a similar level of TR activity on IgG reduction as the Normal group did. In addition, there was no statistical significance between all the experimental groups obtained 1 day after the transient focal ischemia. At post-ischemia day 4, a single increase in the percentage of IgG reduction (7.84 ± 0.69 %) was observed in the ischemic rats with EA stimulation at ST36 (I4-ST) group. Its TR activity was significantly higher than that of the Normal group by 10% ($P<0.05$) and than that of the ischemic rats with EA stimulation at GB20 (I4-GB) group by 14% ($P<0.05$). All the remaining groups did not exhibit remarkably altered TR activities on IgG reduction.

5.3.3 Trx expression

Another component of the thioredoxin system, Trx, was also detected. Its expression patterns obtained from western blotting was shown in Figure 5.3A. Results of their corresponding amounts quantified were then displayed in Figure 5.3B. The basal expression as measured in the Normal group was $0.205 \pm 0.013$ pg Trx per mg protein. Except the Ischemia Day 1-Sham EA-GB20 (I1-SGB) group, the basal Trx expression were up-regulated in all the experimental groups at 1 day or 4 days of transient focal cerebral ischemia ($P<0.05$). In comparison to the ischemia group at day 1 or day 4, Trx expression remained unchanged in those groups with Sham EA stimulation at either GB20 or ST36. However, some interesting findings were observed in those groups with EA stimulation. For example, the expression levels of the I1-GB group was increased to $0.249 \pm 0.016$ pg Trx per mg protein, that was 10% more than that of the I1 group ($P<0.05$). Also, four days after transient focal cerebral ischemia, an upregulated Trx expression were observed in groups with EA stimulation at either GB20 or ST36. Their expressions were over one-fifth greater than those of the
ischemic rats (I4 group) \( (P<0.05) \) and the groups with Sham EA stimulation at each corresponding acupoints \( (P<0.05) \). Besides the significance of treatment delivered to each group, the statistical analysis revealed that the Trx expression was also dependent on the harvest day after cerebral ischemia. Significantly induced Trx expressions were detected when comparing groups with EA stimulation at each acupoint harvested at day 4 to day 1, i.e. I4-GB group to I1-GB group \( (P<0.05) \) and I4-ST group to I1-ST group \( (P<0.05) \). However, there was no observable difference between I1-GB and I1-ST groups and between I4-GB and I4-ST groups, indicating that the EA stimulation at either acupoints may exhibit a similar effect on regulating Trx expression.

5.4 DISCUSSION

In the present study, we first examined the disulphide-reducing activities of TR and the expression level of Trx after transient focal cerebral ischemia. TR activities on the NADPH-dependent direct reduction of small molecules (DTNB) were maintained relatively constant. Similar activity levels were measured on the Trx-mediated reduction of disulphide-containing proteins (IgG molecules). Our findings agreed with the observations that TR was unlikely induced by various stresses [Becker et al., 2000]. However, we found that Trx expression was stress-inducible, which was in line with those previously reported. The induced expression started as early as 1 day and sustained at 4 days after cerebral ischemia-reperfusion injury. Takagi and the collaborators (Takagi et al., 1998) found that Trx mRNA measured by Northern blot was induced at 8 hours and increased until 24 hours of reversible occlusion of middle cerebral artery [Takagi et al., 1998]. Their immunohistochemical studies also
reported that neurons in layer II and V showed the intense Trx immunoreactivity at 24 hour of transient occlusion of middle cerebral artery [Takagi et al., 1998]. Similarly Trx induction was regarded as an early event in head trauma and motor nerve injury that had occurred already 24 hour of injury [Lippoldt et al., 1995; Mansur et al., 1998]. However, a sustainable expression level of Trx had not been reported in cerebral ischemia-reperfusion injury. Only in the motor nerve injury model, Mansur et al. (1998) revealed that the upregulation reached its peak in about 7 days and then gradually decreased after more than 8 weeks [Mansur et al., 1998]. The induction of Trx expression was likely related to the generation of ROS because of the stronger expression of Trx mRNA and proteins in ischemia-reperfusion than in ischemia only [Takagi et al., 1998]. Taken together, we suggested that an ROS-mediated induction of Trx expression was not transient and its induced levels could be maintained for days after injury.

Afterwards, the studies had examined the TR activities and Trx expression on ischemic rats with EA stimulation at either Fengchi (GB20) or Zusanli (ST36). As monitored in the reduction of DTNB or IgG molecules, TR activity was not altered when EA stimulation was applied on GB20, but it was increased at four days after focal cerebral ischemia and EA stimulation on ST36. This increased activity may incorporate with the induced Trx expression to protect the cells from protein disulphide oxidation produced by cerebral ischemia-reperfusion injury. On the other hand, the Trx expression was more susceptible to the EA stimulation on each acupoint. Both induction levels were gradually increasing from post-ischemia day 1 to day 4. Under cerebral ischemia, the reduced form of glutathione (GSH) was being depleted because it was reacted with the activated nitric oxide intermediates to form S-
nitrosoglutathione (GSNO) [Burton, 1985; Gaston et al., 1993]. The depleting level of GSH limited the activities of glutathione peroxidase (GPx) [Luthman and Holmgren, 1982; Takagi et al., 1998], leading to reduced ability on removing ROS intermediates. In our study, over-expression of Trx by EA stimulation could likely restore the active GSH by reducing the GSNO formed [Burton, 1985; Gaston et al., 1993]. Additionally, Trx could replace GSH as the substrate for GPx [Arnér and Holmgren, 2000; Gromer et al., 1999; Lippoldt et al., 1985; Mustacich and Powis, 2000; Nakamura et al., 1994; Poole et al., 2000; Takagi et al., 1998]. Dual actions of Trx probably ensured that GPx was fully active in protecting the cells from injury by hydrogen peroxide. Induced levels of Trx could directly promote the expression of manganese superoxide dismutase (MnSOD), and thereby increase the removal of highly reactive superoxide anions [Das et al., 1997; Gallegos et al., 1996; Nordberg and Arnér, 2001]. Higher removal rate of superoxide anions can effectively protect the cells against nitric oxide injury where the cytotoxic and reactive molecule, peroxynitrate, was formed [Brockhaus and Brune, 1999; Das et al., 1997; Gonzalez-Zulueta et al., 1998; Huie and Padmaja, 1993] and the subsequent induction of iNOS expression [Suzuki et al., 2002]. Such a Trx-directed induction of MnSOD and GPx activity may be regarded as a compensatory increase of the protective enzyme activities in response to the increased oxidative stress [Lippoldt et al., 1995; Lovell et al., 2000].

Moreover, the application of electrical stimulation was necessary to produce the aforesaid neuroprotective effect. Other clinical studies reported that a single application of EA stimulation on GB20, not manual acupuncture, was able to improve blood circulation in cerebral arteriosclerosis [Yu et al., 1998], increase antioxidative
enzyme activities in vascular dementia [Lai et al., 1997], and restore speech ability in stroke patients [Guo et al., 1995; Liu et al., 2000]. In our experiment, neither TR activity nor Trx expression was modified in the ischemic rats with Sham EA stimulation where no electrical stimulation was given to the inserted acupuncture needles. Besides, both acupoints were used in clinical treatment for stroke patients, and produced beneficial effects such as improved motor functions [Liu et al., 1999; Pei et al., 2001; Ruan, 1980; Si et al., 1999; Xu and Cui, 1998], regulated blood flow [Research School of Chinese Medicine, 1979; Xuan et al., 1996; Yuan et al., 1998], and reduce blood viscosity [Chen et al., 1988; Ji et al., 1987; Zhou et al., 1995]. However, some clinical studies found that multiple EA stimulation at GB20 was more effective than ST36 in increasing locomotion [Liu et al., 1999] and restoring speech ability [Zhang and Ni, 1998]. In our study, it was unable to find any difference by using a single EA stimulation at GB20 and ST36 after cerebral ischemia. The variation may partly due to the number of treatment sessions of EA stimulation and partly due to the condition of treatment protocol. Our observations may further suggest that a single application of EA stimulation at bilateral ST36, not only GB20, was possible to produce neuroprotective effects on animal stroke model.

5.5 CONCLUSION
In conclusion, our study showed that EA stimulation either at Fengchi (GB20) or Zusanli (ST36) was able to upregulate the antioxidative activity of Trx system by increasing the availability of Trx to TR.
CHAPTER 6

Effect of electroacupuncture on regulating the apoptosis induced by cerebral ischemia

6.1 INTRODUCTION

Cerebral ischemia-reperfusion triggered the apoptotic cascade that involves the actions of caspase family of proteases and apoptotic proteins. The major causes of induced apoptosis include the massive release of excitotoxic glutamate and the excessive stimulation of the glutamate receptors, and the associated increased production of free radicals [Moroni et al., 2001]. Once it starts, the self-propagated activation of proteases and apoptotic proteins finally leads to the cell death. These events were summarized in Figure 6.1 [Bonfoco et al., 1995; DeGracia et al., 2002; Du et al., 1996; Eldadah and Faden, 2000; Graham and Chen, 2001; Hou and MacManus, 2002; Kirino, 2000; Mattson et al., 2000]. The increase intracellular calcium content directly increases the production of free radicals and indirectly induces the dephosphorylation of BAD protein (Bcl-2-associated death protein) [Rickman et al., 1999] (Stage 1 as indicated in Figure 6.1). The dephosphorylated BAD protein subsequently dimerizes with the antiapoptotic Bcl-XL protein that is constitutively bound to BAX (Bcl-associated X protein) [Henshall et al., 2002] (Stage 2 and 3). During this binding, the displaced BAX protein translocates to the mitochondrion and stimulates the release of cytochrome c [Graham and Chen, 2001] (Stage 4). The cytochrome c promotes the cleavage of pro-caspase-9 into activated caspase-9 that activates the activity of caspase-3 [Eldadah et al., 2000; Graham and Chen, 2001; Harrison et al., 2001; Mouw et al., 2002] (Stage 5 and 6). It finally
results in the impaired energy production and DNA fragmentation, and eventually ends with the cell death [Moroni et al., 2001].

Figure 6.1 Cascade of apoptotic events occurred after ischemia-reperfusion injury.
Although apoptotic pathway is initiated, the brain tissue will induce a series of anti-apoptotic proteins as a defensive mechanism. The phosphatidylinositol 3-kinase/Serine-threonine kinase (PI3-K/Akt) pathway had been regarded as antiapoptotic pathway in various injury models, such as the oxidative stress induced by hydrogen peroxide [Crossthwaite et al., 2002; Neri et al., 2002; Thomas et al., 2002], (Stage 7 as indicated in Figure 6.1). Increased expression of activated phosphorylated Akt by the PI3-K/Akt pathway could directly suppress the dimerization of BAD protein with Bcl-XL and the translocation of BAX protein [Andjelkovic et al., 1997; Dudek et al., 1997; Henshall et al., 2002; Kitagawa et al., 1999; Noshita et al., 2001; Ouyang et al., 1999]. Such an activation of Akt could induce the ischemic tolerance in the CA1 subfield of gerbil hippocampus [Yano et al., 2001]. Another mechanism is the upregulation of Bcl-2 that had been widely reported in traumatic injuries and hypoxia [Hutchison et al., 2001; Jacobson and Raff, 1995; Lee et al., 2001; Martinou et al., 1994; Zhong et al., 1993] (Stage 8 as indicated in Figure 6.1). Increased expression of Bcl-2 could suppress the release of cytochrome c and thereby suppressing the activation of pro-caspase 9 [Seo et al., 2002]. Similarly, Bcl-2 overexpression protects neurons by inhibiting cytochrome c translocation and caspase-3 activity after experimental stroke [Zhao et al., 2003]. Further studies also showed that use of Bcl-2 antisense treatment attenuated the infarction volume [Shimizu et al., 2001]. The last anti-apoptotic mechanism is suggested by increasing the expression of transforming growth factor β-1 (TGFβ-1) (Stage 9 as indicated in Figure 6.1). Ischemic brains transduced with TGFβ-1 promoted the phosphorylation of BAD, resulting in reduced apoptotic bodies observed in brain tissues [Horowitz et al., 2004; Zhu et al., 2002]. Therefore any remedies capable of enhancing the
expression of the aforesaid anti-apoptotic proteins may serve as a possible therapy for stroke.

Electro-acupuncture (EA) has produced a variety of beneficial clinical outcomes to stroke patients, such as reduced speech retardation [Liu *et al.*, 2000; Zhang and Ni, 1998; Zhao *et al.*, 1998], improved locomotion [Liu *et al.*, 1999; Tan, 1990; Zhang and Ni, 1998;], and enhanced memory [Li and Shen, 1998]. EA has also reduced the neurotoxicity of experimentally induced cerebral ischemia by reducing the lipid peroxidation and cerebral infarction as well [OuYang *et al.*, 1999; Xu *et al.*, 1996; Zhao *et al.*, 2000], by inhibiting the production of nitric oxide that initiated the cascade of neuronal damage [Xu *et al.*, 1996; Zhao *et al.*, 2000] and by promoting the scavenging of reactive oxygen species [Siu *et al.*, 2004]. Recently EA has been found to induce the expression of phospho-Akt (pAkt), resulting in the suppression of caspase-9 activation [Wang *et al.*, 2002]. However, it remains unknown if EA stimulation could upregulate the expression of Bcl-2 and TGFβ-1 in ischemia-reperfusion rat brains. In the present study, we thus aimed at investigating the possibility of EA on potentiating expression of anti-apoptotic proteins and suppressing the activities of caspases and expression of apoptotic proteins after transient focal cerebral ischemia.

### 6.2 MATERIALS AND METHODS

#### 6.2.1 Experimental design

Male Sprague-Dawley rats weighing 330-350 g were maintained with free access to food and water under a controlled environment (12 h dark/12 h light cycle, 20-22°C).
Procedures were approved by the Animal Subject Ethics Sub-committee of the Hong Kong Polytechnic University. A total of sixty-six rats were randomly assigned into eleven groups. The induction of cerebral ischemia and EA stimulation given in each group (n=6) were summarized in Table 4.1.

**6.2.2 Induction of focal cerebral ischemia**

Cerebral ischemia by occluding middle cerebral artery (MCAO) was induced according to the procedures as previously described in Section 3.2.4. Rats were anesthetized with an intraperitoneal injection of ketamine and xylazine (Alfasan, Holland). Briefly the temporalis muscle was separated in the plane of its fiber bundles to expose the zygoma and squamosal bone where a hole was made. The exposed right MCA was occluded for 1 h followed by reperfusion for 1 and 4 days.

**6.2.3 Application of electroacupuncture stimulation**

Procedures of EA stimulation were in accordance with those previously described in Section 4.2.3. A single EA stimulation was applied for 30 min following MCAO. Two acupoints commonly used for treating stroke patients, Fengchi (GB20) and Zusanli (ST36), were selected. For the rats receiving Sham EA stimulation at GB20 or EA stimulation at GB20, two stainless steel needles were bilaterally inserted into points corresponding to GB20. For the rats receiving Sham EA stimulation at ST36 or EA stimulation at ST36, two needles were bilaterally inserted into points representing ST36. Electrical stimulation was delivered with EA stimulator (Model G6805-02, Smeif, Shanghai, China) at voltage 0.7 V, frequency 2 Hz, duration 0.5 ms, to the groups receiving EA stimulation at GB20 (I1-GB and I4-GB) and EA
stimulation at ST36 (I1-ST and I4-ST). No electrical stimulation was delivered to ischemic rats with Sham EA stimulation (I1-SGB, I4-SGB, I1-SST and I4-SST).

6.2.4 Chemicals and antibodies

Unless stated otherwise, all materials were of analytical grade and obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Primary antibodies of 200µg/ml were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and used at 1:200 dilution. Purchased primary antibodies included Akt (sc-5298), pAkt (sc-7985), BAD (sc-7869), pBAD (sc-7998), caspase-9 p35 (sc-8355), Bcl-2 (sc-783), TGFβ-1 (sc-146). Internal control using α-tubulin was polyclonal antibody obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibodies conjugated with horseradish peroxidase (HRP) directed against rabbit IgG were obtained from Pierce Chemical Co. (Rockford, IL, USA) and used at a dilution of 1:1000.

6.2.5 Caspase-3 activity assay

The activity assay relies on the catalytic property of caspase-3 that shows specificity for cleavage at the C-terminal side of the aspartate residue of the amino acid sequence DEVD (Asp-Glu-Val-Asp) [Bossy-Wetzel et al., 1998; Thornberry, 1994] (Promega Corporation, Madison, WI, USA). A colorimetric substrate (Ac-DEVD-pNA) is labeled with the chromophore p-nitroaniline (pNA) which is released from the substrate upon cleavage by DEVDase. The activity of caspase-3 is then determined from this release of yellow-coloured free pNA. After 1 day and 4 days of reperfusion, right hemispheres of brains of each group were removed, weighed, homogenized in 2 volumes of ice-cold 20 mM Tris-HCl buffer (pH 7.4) added with protease inhibitor
cocktail (P8340, Sigma, Saint Louis, MO, USA) and phosphatase inhibitor cocktail (P2850, Sigma, Saint Louis, MO, USA). The homogenate was then centrifuged for 30 min at 10,000 x g. The supernatant was collected for protein determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). For the activity assay, 40 µl of supernatant was incubated with 32 µl Caspace-Assay buffer (312.5 mM HEPES, pH 7.5; 31.25% w/v sucrose, 0.3125% w/v CHAPS), 2 µl DMSO, 10 µl 100 mM DTT, 2 µl 10 mM substrate stock (DEVD-pNA) and with dH2O to 100 µl. A pNA standard with a final concentration ranging from 10 µM to 100µM was prepared. After incubation at 37°C for 4 hr, the absorbance of both samples and standards were measured at 405 nm using a microtiter plate reader (Winooski, Vermont, USA). The activity of caspase-3 was determined by the following calculation and its unit was expressed as pmol pNA liberated per hr per mg protein.

\[
\text{Caspase-3 Activity} = \frac{\Delta \text{Abs} \ 405}{4 \text{ hr}} \times \frac{100}{\text{Slope of pNA standard curve}}
\]

### 6.2.6 Expression of apoptosis-related proteins

Amount of apoptosis-related protein were detected from the western blots probed with specific primary antibodies. For the detection of Akt and pAKt, a 10%SDS-PAGE separating gel were prepared. For the detection of other apoptosis-related proteins, a 15% SDS-PAGE was prepared. The western blots were prepared by electro-blotting (25V, 16 h) of a SDS-PAGE separating gel with 200 µg brain homogenate resolved onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Each membrane was rinsed three times with Tris-buffered saline-Tween (TBS-T: 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20) and blocked with 5% skimed...
milk in TBS-T for 2 h at room temperature. Protein blots were individually probed with primary antibody overnight at 4°C. Subsequently, the blots were washed six times (5 mins each) with TBS-T followed by 2-hour incubation with secondary antibodies with gentle shaking. Afterwards, these blots were washed six times with TBS-T for 5 min each. All apoptotic and antiapoptotic proteins were detected by reacting with Supersignal® West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL, USA). Chemiluminescence released was captured by a UVP-Chemi System (UVP, Inc., Upland, CA, USA) and quantified by the Lab-Works™ Image Acquisition and Analysis Software (UVP, Inc., Upland, CA, USA). The unit was defined as pg apoptotic or antiapoptotic protein per mg homogenate protein. Selected blots were re-probed with α–tubulin as internal control.

6.2.8 Statistical analysis

Data were expressed as mean ± standard deviation. Comparisons among different groups were made using multiple analyses of variance (ANOVA) followed by post-hoc protected least-significant different test. In all instances, \( P<0.05 \) was considered statistically significant.

6.3 RESULTS

6.3.1 Expression of Akt and pAkt

Immunoblots using antibodies directed against Akt and phospho-Akt (pAkt) were obtained in Figure 6.2(A) and Figure 6.3(A), and the quantification of expression was shown in Figure 6.2(B) and Figure 6.3(B). As shown in the Normal group, both Akt and pAkt were expressed in normal rats (22.09 ± 1.15 pg Akt per mg protein and 5.79
± 0.83 pg pAkt per mg protein). Both of them were significantly induced by cerebral ischemia-reperfusion (P<0.05). At the post-ischemia day 1, statistical analysis revealed that there was no significance of both protein expressions found in the ischemic with Sham EA stimulation or EA stimulation. Akt expression, was suppressed in I4-ST group at the post-ischemia day 4, when comparing to other corresponding groups (P<0.05). On the other, at post-ischemia day 4, the pAkt expression upregulated to 8.49 ± 0.45 and 8.51 ± 0.603 pg pAkt per mg protein in the I4-SGB and I4-SST group, which were significantly to the I4 group (P<0.05). However, there upregulation values were not significant greater than those in I4-GB and I4-ST groups, suggesting no difference between Sham EA stimulation and EA stimulation on upregulating pAkt levels.

6.3.2 Expression of BAD and pBAD

In Figure 6.4 and Figure 6.5, the western blots showing the abundance of BAD and pBAD were displayed, respectively. Amounts of BAD detected in the experimental groups were not altered from that of the Normal group. Also no statistical difference was found in pBAD expression between the Normal group and all the experimental groups at post-ischemia day 1. Only the I4-ST group expressed significantly increased amount of pBAD (0.282 ± 0.015), which was about 10% more to the I4 and I4-SST groups (P<0.05).
Figure 6.2 Akt expression in normal and operated brains harvested after transient focal ischemia for 1 and 4 days: (A) patterns in immunoblots and (B) the abundance quantified as pg pAkt per mg protein. The data represents mean ± S.D., n=6 in each group. An internal control, α-tubulin, was selectively probed. Symbol (a) represents the significant difference to the Normal group ($P<0.05$). On the same harvest day, symbols (b,c,e) denote the significant differences to the Ischemia group ($P<0.05$), and to the group with corresponding Sham EA stimulation ($P<0.05$), between experimental groups with EA stimulation at two acupuncture points ($P<0.05$), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups ($P<0.05$).
CHAPTER 6: EFFECT OF EA ON REGULATING APOPTOSIS

A

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pAkt (60 KDa)

α-tubulin (54 KDa)

B

Figure 6.3 Phospho-Akt (pAkt) expression in normal and operated brains harvested after transient focal ischemia for 1 and 4 days: (A) patterns in immunoblots and (B) the abundance quantified as pg Akt per mg protein. The data represents mean ± S.D., n=6 in each group. An internal control, α-tubulin, was selectively probed. Symbol (a) represents the significant difference to the Normal group (P<0.05). On the same harvest day, symbols (b) denote the significant differences to the Ischemia group (P<0.05). Symbol (d) represents the significant difference of day of treatment on the same experimental groups (P<0.05).
Figure 6.4  BAD expression in normal and operated brains harvested after transient focal ischemia for 1 and 4 days: (A) patterns in immunoblots and (B) the abundance quantified as pg BAD per mg protein. The data represents mean ± S.D., n=6 in each group. An internal control, α-tubulin, was selectively probed. Statistical analysis revealed no significance between groups.
Figure 6.5 Phospho-BAD (pBAD) expression in normal and operated brains harvested after transient focal ischemia for 1 and 4 days: (A) patterns in immunoblots and (B) the abundance quantified as pg pBAD per mg protein. The data represents mean ± S.D., n=6 in each group. An internal control, α-tubulin, was selectively probed. Statistical analysis revealed no significance between groups. Symbol (a) represents the significant difference to the Normal group (\(P<0.05\)). On the same harvest day, symbols (b,c) denote the significant differences to the Ischemia group (\(P<0.05\)), and to the group with corresponding Sham EA stimulation (\(P<0.05\)), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups (\(P<0.05\)).
6.3.3 Caspase-9 expression

Results of caspase 9 expression obtained in the normal and operated rats were shown in Figure 6.6. In comparison to the ischemic rats, normal rats had a relatively less amount of cleaved caspase 9 expressed (2.556 ± 0.127 pg caspase-9-p35 per mg protein). Induction of cerebral ischemia could significantly increase the amount of cleaved caspase 9 (P<0.05) and the effect was still lasting for 4 days after cerebral ischemia. At post-ischemia day 1, there was no significant reduction on the expression of cleaved caspase 9 when comparing the ischemic rats with EA stimulation and ischemic rats. However, at post-ischemia 4, the expression of cleaved caspase 9 was significantly increased to 7.982 ± 0.21 in ischemic rats (I4 group). This increased expression was effectively decreased in the I4-GB and I4-ST groups, in which the inhibition percentage was kept at 11% and 14 %, accordingly.

6.3.4 Caspase-3 activity

Figure 6.7 showed the results of caspase-3 activity measured in normal and operated rat brain harvested after transient focal ischemia for 1 and 4 days. Caspase 3 activity in ischemic rats of all experimental groups was much higher than that in the Normal group (165.15 ± 7.56 pNA liberated per hr per mg protein) (P<0.05). At post-ischemia day 1, the increased caspase-3 activity was only suppressed by 16% in the I1-GB group (P<0.05). However, at post-ischemia day 4, more than 30% of activity was significantly suppressed in the I4-GB, I4-SST, and I4-ST groups (P<0.05), but not in I4-SGB group.
Figure 6.6 Cleaved Caspase-9 fragment expression in normal and operated brains harvested after transient focal ischemia for 1 and 4 days: (A) patterns in immunoblots and (B) the abundance quantified as pg caspase-9 per mg protein. The data represents mean ± S.D., n=6 in each group. An internal control, α-tubulin, was selectively probed. Symbol (a) represents the significant difference to the Normal group (P<0.05). On the same harvest day, symbols (b,c) denote the significant differences to the Ischemia group (P<0.05), and to the group with corresponding Sham EA stimulation (P<0.05), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups (P<0.05).
Figure 6.7 Caspase-3 activities of normal and operated brains harvested after transient focal ischemia for 1 and 4 days. The activity was determined from liberation of pNA from the DEVD-pNA substrate after specific cleavage by caspase-3. The activity unit is expressed as pmol pNA liberated per hr per mg protein. The data represents mean ± S.D., n=6 in each group. Symbol (a) represents the significant difference to the Normal group ($P<0.05$). On the same harvest day, symbols (b,c,e) denote the significant differences to the Ischemia group ($P<0.05$), and to the group with corresponding Sham EA stimulation ($P<0.05$), between experimental groups with EA stimulation at two acupuncture points ($P<0.05$), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups ($P<0.05$).
6.3.5 Expression of Bcl-2

Immunoblots using antibodies specific for Bcl-2 was obtained in Figure 6.8(A) and the quantification of expression was shown in Figure 6.8(B). Induction of Bcl-2 expression by cerebral ischemia was observed at post-ischemia day 4 (P<0.05). In comparison to the ischemic rats in the I1 and I4 groups, Bcl-2 expression was significantly increased in rats with Sham EA stimulation at GB20 or ST36 (in the I1-SGB, I1-SST, I4-SGB, and I4-SST groups) (P<0.05). EA stimulation at GB20 and ST36 (I1-GB, I1-ST, I4-GB, and I4-ST groups) could further upregulate the Bcl-2 expression, that was significant to the normal and corresponding ischemic rats (P<0.05). However, expression of Bcl-2 was not differed from the ischemic rats with EA stimulation at GB20 to those with EA stimulation at ST36 (i.e. I1-GB vs I1-ST, and I4-GB vs I4-ST).

6.3.6 Expression of TGFβ-1

Expression pattern of TGFβ-1 obtained was shown in Figure 6.9 (A) and the abundance was quantified in Figure 6.9 (B). In the normal rats, TGFβ-1 was minimally expressed (0.324 ± 0.023 pg TGFβ-1 per mg protein). As shown in all the experimental groups, its expression was greatly induced by cerebral ischemia (P<0.05). Further upregulation of TGFβ-1 was only observed in ischemic rats of I4-GB group, I4-SST group and I4-ST group. Their expression was about 12% greater than that of the I4 group (1.558 ± 0.068 TGFβ-1 per mg protein) (P<0.05). However, there was no difference in TGFβ-1 expression found in two groups of with EA stimulation (i.e. I4-GB vs I4-ST).
Figure 6.8 Bcl-2 expression in normal and operated brains harvested after transient focal ischemia for 1 and 4 days: (A) patterns in immunoblots and (B) the abundance quantified as pg Bcl-2 per mg protein. The data represents mean ± S.D., n=6 in each group. An internal control, α-tubulin, was selectively probed. Symbol (a) represents the significant difference to the Normal group \( (P<0.05) \). On the same harvest day, symbols (b,c) denote the significant differences to the Ischemia group \( (P<0.05) \), and to the group with corresponding Sham EA stimulation \( (P<0.05) \), respectively. Symbol (d) represents the significant difference of day of treatment on the same experimental groups \( (P<0.05) \).
Figure 6.9 Transforming growth factor beta-1 (TGFβ-1) expression in normal and operated brains harvested after transient focal ischemia for 1 and 4 days: (A) patterns in immunoblots and (B) the abundance quantified as pg TGFβ-1 per mg protein. The data represents mean ± S.D., n=6 in each group. An internal control, α-tubulin, was selectively probed. Symbol (a) represents the significant difference to the Normal group (P<0.05). On the same harvest day, symbols (b) denote the significant differences to the Ischemia group (P<0.05). Symbol (d) represents the significant difference of day of treatment on the same experimental groups (P<0.05).
6.4 DISCUSSION

The study has first investigated if our animal model of the cerebral ischemia reperfusion induces the apoptosis. Results showed that, 1 day and 4 days after induction of cerebral ischemia, Akt and pAkt expressions were upregulated, and both caspase-3 and caspase-9 were progressively activated. Our findings were partly in line with other observations previously reported. For examples, pAkt upregulation had been reported at the early stage of middle cerebral artery occlusion (with 24 hours) [Friglus et al., 2001; Shibata et al., 2002] and at 4 days of reperfusion [Friglus et al., 2001]. However, Noshita et al. (2003) has reported that the constitutively expressed pAkt was decreased in the ischemic core as early as 1 hour after reperfusion whereas it was temporarily increased in the cortex at 4 hours. Moreover, an amount of pAkt proteins was increased at 3-8 hour in the ischemic penumbra region and declined at 24 hour [Kitagawa et al., 1999]. The activation of caspase-3 occurred as early as 3 hour of reperfusion in the striatum and this activation progressively appeared in the cortex at 24 hour of reperfusion [Davoli et al., 2002; Ferrer et al., 2003; Love et al., 2000; Manabat et al., 2003]. An early activation of caspase-3 has been reported in the penumbral cortex at 4 hour and 6-12 hour following ischemia [Cho et al., 2003; Ferrer et al., 2003; Manabat et al., 2003]. However, caspase-3 activity was also found to be elevated 24 hour and 72 hour after 2-hour occlusion of middle cerebral artery and reperfusion [Lee et al., 2002]. Caspase 9 activation was found to be observed after 24 hour of transient middle cerebral artery occlusion [Cho et al., 2003; Zhang et al., 2002]. On the other hand, our results revealed that the Bcl-2 was upregulated at post-ischemia day 1 and TGFβ-1 was induced at 1 day and 4 days of cerebral ischemia. It seems that Bcl-2 was only transiently induced, which paralleled to the findings that the maximal Bcl-2 expression was observed within 6 hours of middle cerebral
ischemia [Gillardon et al., 1996, Numata et al., 2002]. The consistent expression of TGFβ-1 in our study was agreed with the observation that its expression level was maintained persistently from 2 to 15 days after cerebral ischemia [Wang et al., 1995; Yamashita et al., 1999]. Nevertheless, there was upregulation of BAD and pBAD protein found in our results, which was differed from the induction of BAD protein reported previously [Richman et al., 1999]. Despite the slight variations of the findings, our ischemic model employing 1-hour occlusion of middle cerebral artery was sufficient enough to induce the apoptosis [Kilic et al., 2002]

In this study, EA stimulation has shown several upregulations on the expression of anti-apoptotic proteins and downregulation of apoptotic proteins and caspases. For examples, the expression of phosphorylated pAkt and Bcl-2 was increased in using EA stimulation at either acupoints after 1 day and 4 day of cerebral ischemia. TGFβ-1 expression was only upregulated 4 days after cerebral ischemia. However, decreased Akt expression was only observed in ischemic rats with EA stimulation at ST36 after 4 days. Decreased caspase-3 activity together with suppressed caspase-9 activation consistently occurred in day 4 after EA stimulation at either GB20 or ST36. The upregulation of Akt and the suppression of caspase-9 were consistent with the findings previously reported [Wang et al., 2002]. As observed in this study, the increased Bcl-2 and TGFβ-1 may also serve to increase the suppression of apoptosis. Increased Bcl-2 protein expression could decrease the formation of peroxynitrite or nitric oxide induced during cerebral ischemia [Lee et al., 2001; Seo et al., 2002], and reduce the cerebral infarct volume [Cao et al., 2002]. Upregulation of TGFβ-1 could upregulate the expression of brain-derived neurotrophic factor (BDNF) [Zhu et al., 2001] and inhibit the apoptotic cascade induced by NO via increasing Bcl-2 protein.
expression [Flanders et al., 1998; Prehn, et al., 1994; Zhu et al., 2001]. Taken overall, EA stimulation may produce antiapoptotic effect by different regulation on the apoptotic pathway. A schematic diagram illustrating this assumption was presented in Figure 6.9. Firstly, the induction of TGFβ-1 could effectively increase the phosphorylation of BAD [Zhu et al., 2002]. Secondly, the increased expression of pAkt could effectively suppress the dimerization of BAD and Bcl-XL and the translocation of BAX [Wang et al., 2002]. In response to either increased free radical production or to the upregulation of TGFβ-1 protein [Flanders et al., 1998; Prehn, et al., 1994; Zhu et al., 2001], induced Bcl-2 expression could inhibit the release of cytochrome c and thereby suppress the activation of caspase-9.

Similar to the findings in Chapter 5, the application of electrical stimulation was essential to produce the aforementioned neuroprotective effect [Yuan et al., 1998]. Sham EA stimulation at either acupoints could not modulate the activation of caspase-3 and caspase-9 as induced by the cerebral ischemia. On the other hand, it was hard to compare the effectiveness of EA stimulation at GB20 and ST36 after cerebral ischemia. Both produced comparable amount of modulated protein expression in apoptosis, which was consistent with the clinical findings that both can produce beneficial effect on stroke patients [Chen et al., 1988; Ji et al., 1987; Liu et al., 1999; Pei et al., 2001; Ruan, 1980; Si et al., 1999; Xu and Cui, 1998; Xuan et al., 1996; Yuan et al., 1998; Zhou et al., 1995].
Figure 6.10 (A) Apoptotic events occurred after cerebral ischemia-reperfusion, and (B) Apoptotic events occurred after the cerebral ischemia-reperfusion followed by EA stimulation.
To sum up, according to the events occurred after ischemia-reperfusion illustrated in Figure 6.10(A) (modified from Figure 6.1), the beneficial effect of EA stimulation, particularly EA stimulation at GB20 or ST36, were presented in a schematic diagram in Figure 6.10(B). Firstly, after EA stimulation, the dephosphorylation of pBAD was inhibited by the induced expression of TGFβ-1 (Stage 1 and 9 as indicated in Figure 6.10(B)). Simultaneously, the increased expression of activated Akt (pAkt) could inhibit the BAD proteins from forming dimers with the Bcl-XL and displacing the BAX protein (Stage 7). However, the BAD proteins detected in the study were remaining relative constant (Stage 2). The decreased BAX was likely associated and the translocation of BAX to the mitochondria was reduced (Stage 3). Together with the suppressing effect by the Bcl-2, therefore, the cytochrome c release from mitochondria was reduced (Stage 4 and 8). In this regard, the caspase-9 activation was suppressed, leading to the decreased activity of caspase-3 (Stage 5 and 6). As a whole, the apoptotic effect was minimized.

6.5 CONCLUSION

EA stimulation at either GB20 or ST36 was found to induce the expression of antiapoptotic proteins, including the TGFβ-1, pAkt and Bcl-2, resulting to the reduction of caspase-3 activity and expression of activated caspase-9. The mechanism of this modulation was hypothesized. The necessity for the electrical stimulation has been demonstrated.
CHAPTER 7

Effect of delayed post-ischemia electroacupuncture stimulation

7.1 INTRODUCTION

Nitric oxide, being the fast producing reactive oxygen species, has been implicated in the neurotoxicity of cerebral ischemia-reperfusion injury [Iadecola, 1997; Kader et al., 1993]. The huge production of NO is in parallel to the upregulated expression of NOS isoforms [Holtz et al., 2001]. In particular, the NO produced by upregulated nNOS and iNOS are detrimental to neurons, whereas the NO produced by eNOS is cerebroprotective [Leker et al., 2001; Zhang et al., 1993]. Previous studies found that use of specific inhibitors could reduce the cerebral infarction and hence attenuate the damaging effects of NO [Nagafuji et al., 1995b; Yoshida et al., 1994; Zhang et al., 1996a]. Therefore any treatment capable of down-regulating NOS expression or inhibiting NOS activity may serve as a possible treatment strategy for the stroke.

As mentioned previously, electro-acupuncture (EA) has produced a variety of beneficial clinical outcomes to stroke patients, such as reduced speech retardation [Liu et al., 2000; Zhang and Ni, 1998; Zhao et al., 1998], improved locomotion [Liu et al., 1999; Tan, 1990; Zhang and Ni, 1998], and enhanced memory [Li and Shen, 1998]. EA has also reduced the neurotoxicity of experimentally induced cerebral ischemia by reducing the lipid peroxidation and cerebral infarction as well [OuYang et al., 1999; Xu et al., 1996; Zhao et al., 2000], by inhibiting the production of nitric oxide that initiated the cascade of neuronal damage [Xu et al., 1996; Zhao et al., 2000] and by promoting the scavenging of reactive oxygen species [Siu et al., 2004].
However, the use of EA stimulation was only restricted to apply immediately after the
induction of cerebral ischemia. The possible effect of delayed EA stimulation on
reducing cerebral infarction has only been seldom investigated [Du and Di, 2000;
Wang and Wang, 2001]. In this preliminary study, we thus aimed at investigating the
possibility of delayed EA stimulation on regulating NOS activity and expressions so
as to reduce the extent of lipid peroxidation, with the comparison to the immediate
EA stimulation following cerebral ischemia-reperfusion.

7.2 MATERIALS AND METHODS

7.2.1 Experimental design

Male Sprague-Dawley rats weighing 330-350 g were maintained with free access to
food and water under a controlled environment (12 h dark/12 h light cycle, 20-22°C).
All experimental procedures described below were approved by the Animal Subjects
Ethics Sub-committee of The Hong Kong Polytechnic University. A total of 38 rats
were assigned into six groups: Normal (n=8), Ischemia (n=8), Ischemia with
immediate EA stimulation (I0D, n=8), Ischemia with 1-day delayed EA stimulation
(I1D, n=4), Ischemia with 2-day delayed EA stimulation (I2D, n=5), and Ischemia
with 3-day delayed EA stimulation (I3D, n=5).

7.2.2 Induction of focal cerebral ischemia

Transient focal cerebral ischemia was induced by occlusion of right middle cerebral
artery (MCA) as previously described in Section 3.2.4. Briefly, after the ketamine-
xylazine anesthesia, the temporalis muscle was separated in the plane of its fiber
bundles to expose the zygoma and squamosal bone. A 5 mm x 5 mm burr hole was made to expose the right MCA, which was occluded for 1 h followed by reperfusion.

**7.2.3 Application of electroacupuncture stimulation**

A single application of 30-minute EA stimulation at Fengchi (GB20) by EA stimulator (Model G6805-02, Smeif, Shanghai, China: Voltage 0.7V; Frequency 2Hz; Duration 0.5ms) was given to groups designed with EA stimulation. For the groups with delayed EA stimulation, EA was given immediately, 1 day, 2 day or 3 day after the induction of transient focal cerebral ischemia. Rats were then harvested at post-ischemia day 4.

**7.2.4 Assay of total NOS activity**

NOS activity was determined by the total amount of NO produced from L-arginine by Griess reaction as previously described in Section 3.2.8. In short, one and four days after ischemia-reperfusion, the perfused right brains were harvested, weighed and homogenized mechanically. After centrifugation (10,000 x g, 30 min, 4°C), the supernatant was taken for 2-hour incubation with all cofactors. The incubated mixture was subsequently taken for the determination of NO according to the Griess reaction and the absorbance of end-products was measured at 540 nm. The total NOS activity was expressed as pmol nitrate produced per hour per mg protein.

**7.2.5 Expression of NOS isoforms**

Amount of NOS isoforms were detected from the western blots probed with specific primary antibodies. Procedures were in accordance with those previously described in Section 3.2.9. A 10% and 15% SDS-PAGE separating gel were prepared for the detection of NOS isoforms and SOD isoforms, respectively. In brief, homogenate
protein (200 µg) resolved by gel electrophoresis were electroblotted to a nitrocellulose membrane at a constant voltage 25 V for 16 hr. After transfer, membranes were blocked with skimmed milk in 1X TBS-T and then probed with primary antibody raised against nNOS, eNOS iNOS overnight at 4°C (ordered from Santa Cruz Biotechnology, Inc., Santa Cruz, USA and used at 1:200 dilution). Subsequently, the probed blots were incubated with secondary antibodies with gentle shaking. The isoforms of NOS were detected by reacting with chemiluminescent substrate (Pierce Chemical Co., Rockford, IL, USA). Chemiluminescence released was captured and quantified as pg NOS isoform per mg protein. Selected blots were re-probed with β−actin as an internal control.

7.2.6 Lipid peroxidation assay

Malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE) was determined from the reaction with a chromogenic reagent to yield a stable products with maximum absorbance at 586 nm (Oxis International, Inc., Portland, OR, USA) [Erdelmeier et al., 1998]. Procedures were described in Section 3.2.12. The unit was defined as [MDA] (µM) per mg homogenate protein or [MDA + 4-HNE] (µM) per mg homogenate protein.

7.2.7 Statistical analysis

Data were expressed as mean ± standard deviation. Comparison among these groups were made using analysis of variance (ANOVA) followed by post-hoc protected least-significant difference test. In any cases, it is considered to be statistically significant when P<0.05.
7.3 RESULTS

7.3.1 NOS activity and expression

Total NOS activity measured at day 4 after cerebral ischemia was shown in Figure 7.1, and the abundance of NOS isoforms was obtained in Figure 7.2 to Figure 7.4. The Normal group had the total NOS activity at 290.43 ± 43.16 pmol nitrate produced per hr per mg protein. After reperfusion for 4 day, the NOS activity was doubled to give 569.67 ± 68.66 pmol nitrate produced per hr per mg protein. This increased NOS activity was suppressed by the EA stimulation at Fengchi regardless of the time of EA stimulation. All the ischemic rats with immediately EA stimulation and delayed EA stimulation reduce the increased NOS activity to the normal level by about 60% (P<0.05).

Abundance of NOS isoforms was almost consistent with that in the total NOS activity. In the Normal group, the amount of expressed isoforms were 222.29 ± 7.03 pg nNOS per mg protein, 75.66 ± 9.76 pg iNOS per mg protein and 119.34 ± 4.97 pg eNOS per mg protein. Upon the induction of cerebral ischemia, the expression was increased to 468.28 ± 48.4 pg nNOS per mg protein, 177.32 ± 22.36 pg iNOS per mg protein and 236.48 ± 36.21 pg eNOS per mg protein. These increased expression were significant to that of the Normal group (P<0.05). According to the nNOS expression, use of immediate EA stimulation following the cerebral ischemia could give the lowest nNOS expression which was significantly different from those of the ischemic rats with 2-day and 3-day delayed EA stimulation (I2D and I3D groups) (P<0.05), but not from the ischemic rats with 1-day delayed EA stimulation (P>0.05). Moreover, the I2D and I3D groups showed over 25% increase in nNOS proteins in comparison to the Normal group (P<0.05). However, the iNOS expression was dependent on the
length of delayed time with EA stimulation. The ischemic rats with 2-day delayed 
EA stimulation produced the lowest expression (25.71 ± 9.77 pg iNOS per mg protein) 
and this expression was maintained in ischemic rats with 3-day delayed EA 
stimulation (30.21 ± 9.48 pg iNOS per mg protein). Their expressions were 
significantly less than those of the Normal group and other experimental groups 
(P<0.05). In contrast, the increase in length of delayed EA stimulation promoted the 
eNOS expression. The ischemic rats with 3-day delayed EA stimulation gave the 
highest eNOS expression (172.26 ± 29.57 pg eNOS per mg protein), which was 
significant to the Normal group and other experimental groups (P<0.05).

7.3.2 Lipid peroxidation

Levels of lipid peroxidation, in terms of MDA and 4-HNE, measured were shown in 
Figure 7.5. There were 26.19 ± 4.2 µM MDA per mg protein and 86.62 ± 3.85 µM 
MDA+4-HNE per mg protein detected in the Normal group. Upon the induction of 
cerebral ischemia, MDA and MDA+4-HNE were dramatically increased to 72.06 ± 
8.23 µM MDA per mg protein and 151.65 ± 9.53 µM MDA+4-HNE per mg protein at 
day 4 (P<0.05). Use of EA stimulation at GB20 did not reduce their contents because 
all the groups were detected with comparable values of lipid peroxidation products 
(P>0.05).
Figure 7.1  Total nitric oxide synthase (NOS) activity in normal rat brains and rat brains induced with cerebral ischemia and delayed EA stimulation. Data represents mean ± standard deviation. Sample size: n=8 (Normal, Ischemia and I0D); n=4 (I1D); and n=5 (I2D and I3D). Unit is defined as pmol nitrate produced per hour per mg protein. Symbols (a, b) indicate the significant differences in comparison to Normal (P<0.05), and to Ischemia (P<0.05), respectively.
Figure 7.2  Expression of neuronal nitric oxide synthase (nNOS) in normal rat brains and rat brains induced with cerebral ischemia and delayed EA stimulation: (A) selected western blot using antibodies specific for nNOS of the experimental groups with β-actin as internal control; (B) Quantification of nNOS detected. Data represents mean ± standard deviation. Sample size: n=8 (Normal, Ischemia and I0D); n=4 (I1D); and n=5 (I2D and I3D). Unit is defined as pg nNOS expressed per mg protein. Symbols (a, b, c) indicate the significant differences in comparison to Normal (P<0.05), to Ischemia (P<0.05), to Ischemia with immediate EA stimulation (I0D) (P<0.05), respectively.
Figure 7.3  Expression of inducible nitric oxide synthase (iNOS) in normal rat brains and rat brains induced with cerebral ischemia and delayed EA stimulation: (A) selected western blot using antibodies specific for iNOS of the experimental groups with β-actin as internal control; (B) Quantification of iNOS detected. Data represents mean ± standard deviation. Sample size: n=8 (Normal, Ischemia and I0D); n=4 (I1D); and n=5 (I2D and I3D). Unit is defined as pg iNOS expressed per mg protein. Symbols (a, b, c) indicate the significant differences in comparison to Normal (P<0.05), to Ischemia (P<0.05), to Ischemia with immediate EA stimulation (I0D) (P<0.05), respectively.
Figure 7.4 Expression of endothelial nitric oxide synthase (eNOS) in normal rat brains and rat brains induced with cerebral ischemia and delayed EA stimulation: (A) selected western blot using antibodies specific for eNOS of the experimental groups with β-actin as internal control; (B) Quantification of eNOS detected. Data represents mean ± standard deviation. Sample size: n=8 (Normal, Ischemia and I0D); n=4 (I1D); and n=5 (I2D and I3D). Unit is defined as pg eNOS expressed per mg protein. Symbols (a, b, c) indicate the significant differences in comparison to Normal (P<0.05), to Ischemia (P<0.05), to Ischemia with immediate EA stimulation (I0D) (P<0.05), respectively.
Figure 7.5 Levels of lipid peroxidation products measured in normal rat brains and rat brains induced with cerebral ischemia and delayed EA stimulation: (A) only malondialdehyde (MDA); (B) MDA + 4-HNE (4-hydroxy-2-(E)-nonenal): Data represents mean ± standard deviation. Sample size: n=8 (Normal, Ischemia and I0D); n=4 (I1D); and n=5 (I2D and I3D). Unit is defined as [MDA] (µM) or [MDA + 4-HNE] (µM) per mg protein. Symbol (a) indicates the significant difference in comparison to Normal (P<0.05).
7.4 DISCUSSION

Under cerebral ischemia-reperfusion injury, the cessation of blood flow and deprivation of nutrients induced the activation of glutamate receptors, the increase in Ca\textsuperscript{2+} influx into intracellular compartment of neurons and the activation of NOS expression [Clavier et al., 1994; Samdani et al., 1997; Zhang et al., 1995]. nNOS, whose expression was being Ca\textsuperscript{2+}-dependent, was induced vigorously [Samdani et al., 1997]. It was also observed in this preliminary study that the nNOS expression was upregulated in day 4 after cerebral ischemia-reperfusion. The induced nNOS in turns triggered the iNOS expression via the transcriptional factor nuclear factor kappaB (NFκB) [Togashi et al., 1997]. Our results also detected the high expression of iNOS after cerebral ischemia–reperfusion. The increase eNOS expression may serve as a compensatory role in response to the increased nNOS and iNOS expression [Iadecola, 1997].

Reduction of NO production by EA stimulation has been reported [Zhao et al., 2000]. In addition, the results as obtained in Chapter 4 found that the EA stimulation at GB20 and ST36 could regulate the NO production. As the EA stimulation was delivered immediately following the cerebral ischemia, we then investigated whether the time to deliver EA stimulation would affect the regulation of NOS activity and expression. According to the result, NOS activity was suppressed in a similar manner that no significant difference was found among the groups with immediately EA stimulation and delayed EA stimulation. nNOS and eNOS expression was dependent on the time of EA stimulation, i.e. increase in the delayed period to deliver EA stimulation resulted in higher expression levels. The decreased nNOS activity may be possibly due to the suppressed release of excitatory amino acids that triggered the
influx of extracellular calcium [Zhao et al., 1997]. It hence inhibited the calcium-dependent nNOS expression. This increased eNOS expression may be as a result of the EA-stimulated increase of the amount of basic fibroblast growth factor (bFGF) as reported by Ou et al. (2001). Since the activity of superoxide dismutase (SOD), particularly the copper-zinc superoxide dismutase (CuZnSOD), was not measured, it was not able to postulate if the eNOS upregulation was induced by CuZnSOD [Brennan et al., 2002; Brennan et al., 2003; Kim et al., 2001]. On the other hand, the down-regulation of iNOS expression was associated with longer delayed period to deliver EA stimulation. This suppressed expression may be related to the down-regulated mRNA and protein expression of interleukin-1 (IL-1) after EA stimulation [Xu et al., 2002; Zhang et al., 1994]. Taken as a whole, the lipid peroxidation levels were not altered in ischemic rats with and without EA stimulation. Usually the decreased iNOS expression and enhanced eNOS expression would lead to reduced cerebral infarction as well as lipid peroxidation [Iadecola et al., 1995a; Iadecola et al., 1996; Zhang et al., 1996b]. The deviation as observed in this study may suggest that some other proteins, especially the antioxidative enzymes (SOD and glutathione peroxidase), were not suppressed by the delayed EA stimulation at GB20. The suppression would lead to accumulation of superoxide anion that was in favour of the production of peroxynitrite [Gonzalez-Zulueta et al., 1998; Huie and Padmaja, 1993] which could extensively trigger the lipid peroxidation [Watson et al., 1996]. Therefore, further studies recruiting the measurement of activities or expression of antioxidative enzymes are suggested.
7.5 CONCLUSION

To conclude, the preliminary study shows that the NOS activity and the lipid peroxidation were not altered by the time to deliver EA stimulation. However, the specific immunoblots found that nNOS and eNOS was upregulated while the iNOS was down-regulated, with time to deliver EA stimulation. The underlying pathway may not become clear unless the results from the antioxidative enzymes were obtained and larger sample size was recruited.
CHAPTER 8

Effectiveness of multiple pre-ischemia electroacupuncture on regulating neuronal damage induced by cerebral ischemia

8.1 INTRODUCTION

Reactive oxygen species has been implicated in the neuronal injury. Nitric oxide (NO), being produced within minutes, is one of the first reacting free radicals produced by this mechanism during cerebral ischemia [Iadecola, 1997]. It is generated by a two-step conversion of L-arginine at the guanidine group and is catalyzed by an enzyme called nitric oxide synthase (NOS) [Moncada et al., 1991]. There are at least three NOS isoforms: neuronal NOS (nNOS) [Bredt et al., 1990; Schmidt et al., 1991], inducible NOS (iNOS) [Yui et al., 1991] and endothelial NOS (eNOS) [Pollock et al., 1991]. Briefly, NO produced by nNOS and iNOS induce the neuronal cell death, while NO produced by eNOS is cerebroprotective as it improves the cerebral blood flow in situ [Leker et al., 2001; Zhang et al., 1993]. However the beneficial effects of endothelial NO cannot outweigh the neurotoxic potentials of neuronal and inducible NO produced [Iadecola, 1997]. Therefore, taken overall, NO produced during cerebral ischemia is predominantly neurotoxic [Zhang et al., 1994]. As NO contributes to the pathogenesis of ischemic brain injury, regulation of NO generation becomes a possible strategy for treatment of stroke.

Transforming growth factor beta-1 (TGFβ-1) is a ubiquitous cytokine that exerts biological effects on a variety of cell types, including microglia [Lehrmann et al., 1995] and neurons [Henrich-Noack et al., 1996; Lehrmann et al., 1995]. It is virtually
absent in the intact brain [Henrich-Noack et al., 1996; Lehrmann et al., 1998], but its expression increases strongly after trauma [Henrich-Noack et al., 1996], excitotoxic lesioning [Lehrmann et al., 1995] and ischemia-reperfusion injury [Wang et al., 1995]. Induction of TGFβ-1 can produce several anti-inflammatory effects, including decreasing the neuronal susceptibility to glutamate excitotoxicity [Lehrmann et al., 1998], deactivating the macrophages [Lehrmann et al., 1998], and inducing expression of brain-derived neurotrophic factor (BDNF) [Lehrmann et al., 1998]. TGFβ-1 also contributes to the calcium homeostasis in nerve cells that is important to regulate the expression of nNOS [Lehrmann et al., 1995] and reduce the iNOS expression [Thomas et al., 2001] induced by cerebral ischemia-reperfusion. Therefore, the increases in TGFβ-1 protein may serve as an initiator of neuroprotective mechanism.

Lipid peroxidation is triggered by high levels of free radicals which can be induced in the cerebral ischemia-reperfusion model [Cheeseman and Slater, 1993; Esterbauer et al., 1987; Esterbauer et al., 1991; Romero et al., 1998]. Determination of its products, malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE), in brain tissues and plasma had been used to evaluate the severity of neuronal injury of ischemia [Bromont et al., 1989; Kondo et al., 1997; Re et al., 1997]. Both MDA and 4-HNE are cytotoxic to cells but their reactivities to biomolecules are different [Cheeseman and Slater, 1993]. MDA demonstrates its specific reactivity on proteins, while 4-HNE has its specificity on DNA and membrane, thus inducing apoptosis [Choudhary et al., 2002]. For examples, at a concentration of 4 mM, MDA nearly completely inhibited the total enzyme activity of xanthine oxidoreductase, whereas HNE could partially inhibit it [Cighetti et al., 2001]. On the other hand, only 4-HNE was
effectively able to decrease membrane fluidity and inhibit the mitochondrial transcription at millimolar concentration [Chen and Yu, 1994; Kristal et al., 1994].

Acupuncture, in particular, electro-acupuncture (EA) has been more recommended as the 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; Wu and Cao, 1998]. A considerable number of studies had been conducted on investigating the effectiveness of EA therapy on patients with cerebral ischemia. Several beneficial outcomes were observed, such as reduced paralysis by increasing muscle strength [Huang et al., 1998; Xing and Zhang, 1998], improved speech ability [Xing and Zhang, 1998], reduced mental retardation [Xing and Zhang, 1998], restored cerebral blood flow [Di et al., 1993; Tan, 1990] and improved locomotion [Chang et al., 1996; Liu et al., 1999; Tan, 1990; Xing and Zhang, 1998]. These observations support the hypothesis that post-ischemic EA can be an effective modality of treatment in stroke condition. However, little is known about the effectiveness of pre-ischemic EA on minimizing the neurological injury caused by stroke. Our experimental study, therefore, aims to investigate the effect of multiple applications of EA stimulation before the induction of cerebral ischemia. There are two acupoints selected for this study, Fengchi (GB20) and Zusanli (ST36). Fengchi, which has been an effective acupoint selected for stroke rehabilitation, has produced a greatly enhanced locomotion ability of stroke patients [Liu et al., 1999; Tan, 1990]. On the other hand, Zusanli is regarded as a common acupoint treating various diseases [Yun et al., 2002]. In order to address the significance of selection of correct acupoints, bilateral non-acupoints were additionally selected for comparison. After the induction of cerebral ischemia, outcomes of EA stimulation on different acupoints
were assessed by measuring the NOS activity, NOS expressions of NOS isoforms, TGFβ-1 and Bcl-2 and by quantifying the amount of both products of lipid peroxidation, MDA and 4-HNE.

8.2 MATERIALS AND METHODS

8.2.1 Experimental conditions

A total of 68 male Sprague-Dawley rats 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. Secondly, eight rats were assigned as the Normal group for measurement of parameters listed in below, except that four rats were taken for measurement of lipid peroxidation. Forty-eight rats were evenly assigned to four experimental groups: (1) Control group (rats with anaesthesia but without EA), (2) Non-acupoint (NA) group (rats with EA stimulation on non-acupoint), (3) GB20 group (rats with EA stimulation on Fengchi, GB20), and (4) ST36 group (rats with EA stimulation on Zusanli, ST36). EA stimulation was carried out on alternative days before the induction of cerebral ischemia. In each group, six rats were subjected to 3 times of EA conducted for a week and another six rats were subjected to 18 times of EA conducted in 6 consecutive weeks. Rats in the experimental groups were then induced with transient focal cerebral ischemia, followed by harvest of brains at post-ischemia day 4.

8.2.2 Pre-ischemia Electro-acupuncture

A thirty-minute EA was applied by an acupunctoscope device (Model G6805-2, Smeif, Shanghai, China) (Voltage: 0.7 V, frequency: 2Hz, duration: 0.5ms) as
previously described in Section 4.2.3. In the GB20 and ST36 groups, EA was applied on bilateral points corresponding to GB20 or ST36, respectively. GB20 is anatomically located on the posterior aspect of the neck, below the occipital bone, in the depression between the sternocleidomastoid muscle and trapezius muscle [Ellis et al., 1991]. ST36 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 on non-acupoint which was located midway between coccyx and hip joints. Figure 8.1 illustrates the location of acupoints used.

8.2.3 Production of transient focal cerebral ischemia

Transient focal cerebral ischemia was induced by occlusion of right middle cerebral artery (MCA) for 1 hour as previously described in Section 3.2.4. Rats were anesthetized with intraperitoneal injection of ketamine and xylazine (Alfasan, Holland). Briefly the temporalis muscle was separated in the plane of its fiber bundles to expose the zygoma and squamosal bone. A 5 mm x 5 mm burr hole was made to expose the MCA that was then occluded for 1 hour followed by reperfusion. During the surgical procedures, rectal temperature of rats was monitored and maintained at about 37°C with an overhead lamp. After recovery from experimental surgery, all rats were allowed to recover at ambient temperature (21-23°C) with food and water made available ad libitum until harvest. For the measurement of physiological parameters, arterial blood samples were taken from the femoral artery 15 min before and after MCA occlusion for the determination of pH, arterial partial pressure of oxygen (paO₂) and carbon dioxide (paCO₂) by a Blood Gas and Electrolyte System (Radiometer ABL505, Copenhagen, Denmark).
Figure 8.1. Rats receiving electroacupuncture on non-acupoints in the NA group, Fengchi in the GB20 group, and Zusanli in the ST36 group before cerebral ischemia.
8.2.4 Chemicals and antibodies

Unless stated otherwise, all materials were of analytical grade and obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Primary antibodies of 200 µg/ml probed against nNOS, iNOS, eNOS, TGFβ-1, and Bcl-2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and used at 1:200 dilution. Internal control using β-actin was monoclonal antibody obtained from Sigma Chemical Co. (A5441; dilution 1:2000). Secondary antibodies conjugated with horseradish peroxidase (HRP) directed against rabbit IgG and mouse IgG were obtained from Pierce Chemical Co. (Rockford, IL, USA) and used at a dilution of 1:1000 and 1:5000, respectively. Protein concentration was determined by using protein assay as described by Bradford (1976) (Bio-Rad Laboratories, Hercules, CA, USA).

8.2.5 Assay of total NOS activity

NOS activity was determined by the total amount of NO produced from L-arginine by Griess reaction as previously described in Section 3.2.8. In short, one and four days after ischemia-reperfusion, the perfused right brains were harvested, weighed and homogenized mechanically. After centrifugation (10,000 x g, 30 min, 4°C), the supernatant was taken for 2-hour incubation with all cofactors. The incubated mixture was subsequently taken for the determination of NO according to the Griess reaction and the absorbance of end-products was measured at 540 nm. The total NOS activity was expressed as pmol nitrate produced per hour per mg protein.

8.2.6 Expression of NOS isoforms, TGFβ-1, Bcl-2

Amount of NOS isoforms, TGFβ-1 and Bcl-2 were detected from the western blots probed with specific primary antibodies. Procedures were in accordance with those
previously described in Section 3.2.9. A 10% and 15% SDS-PAGE separating gel were prepared for the detection of NOS isoforms, and TGFβ-1 and Bcl-2, respectively. In brief, homogenate protein (200 µg) resolved by gel electrophoresis were electroblotted to a nitrocellulose membrane at a constant voltage 25 V for 16 hr. After transfer, membranes were blocked with skimmed milk in 1X TBS-T and then probed with primary antibody raised against NOS isoforms, TGFβ-1 and Bcl-2 overnight at 4°C with gentle shaking. Subsequently, the probed blots were incubated with secondary antibodies with gentle shaking. The immunolabelled proteins (isoforms of NOS or SOD) were detected by reacting with chemiluminescent substrate (Pierce Chemical Co., Rockford, IL, USA). Chemiluminescence released was captured and quantified as pg labeled proteins per mg protein. Selected blots were re-probed with β–actin as an internal control.

8.2.7 Lipid peroxidation assay
Malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE) was determined from the reaction with a chromogenic reagent to yield a stable products with maximum absorbance at 586 nm (Oxis International, Inc., Portland, OR, USA) [Erdelmeier et al., 1998]. Procedures were described in Section 3.2.12. The unit was defined as [MDA] (µM) per mg homogenate protein or [MDA + 4-HNE] (µM) per mg homogenate protein.

8.2.8 Statistical analysis
Data were expressed as mean ± standard deviation. Statistical analysis of physiological parameters was performed using the paired t-test (SPSS version 11.0, Chicago, IL, USA). Multivariate analysis of the other parameters found a significant
interaction between two factors, selection of acupoints and number of pre-ischemic EA stimulation. Therefore, comparison among these groups were made using analysis of variance (ANOVA) followed by post-hoc protected least-significant difference test. In any cases, it is considered to be statistically significant when $P<0.05$.

8.3 RESULTS

8.3.1 Physiological parameters

The physiological data obtained before and during transient focal cerebral ischemia are shown in Table 8.1. Values of the parameters measured were consistent with those previously reported [Leker et al., 2001, Siu et al., 2004]. The difference between $p_{aO_2}$ measured before ($104.75 \pm 10.76$ mmHg) and after ($107.80 \pm 6.67$ mmHg) ischemia was not statistically significant ($t=0.846$, degrees of freedom (d.f.)=5, $\alpha=0.05$).

Similarly, $p_{aCO_2}$ measured before ischemia, $43.45 \pm 3.5$ mmHg, was comparable to that after MCA occlusion, $44.30 \pm 5.06$ ($t=0.270$, d.f.=5, $\alpha=0.05$). In addition, no significant difference of blood pH was observed before ($7.229 \pm 0.029$) and after ($7.223 \pm 0.039$) cerebral ischemia. These findings suggested the observed changes in cerebral tissues were likely due to the challenge induced by ischemia-reperfusion, instead of acidosis [Siu et al., 2004].
Table 8.1. Physiological data from rats subjected to transient focal cerebral ischemia.

<table>
<thead>
<tr>
<th></th>
<th>paO$_2$ (mmHg)</th>
<th>paCO$_2$ (mmHg)</th>
<th>pH (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-ischemia</td>
<td>104.75 ± 10.76</td>
<td>43.45 ± 3.5</td>
<td>7.229 ± 0.029</td>
</tr>
<tr>
<td>(n= 6)</td>
<td></td>
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<tr>
<td>Post-ischemia</td>
<td>107.8 ± 6.67</td>
<td>44.30 ± 5.06</td>
<td>7.223 ± 0.039</td>
</tr>
<tr>
<td>(n= 6)</td>
<td></td>
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Data (mean ± one standard deviation) obtained from arterial blood samples in rats at pre-ischemia 15 min and after 15 min of ischemia. Abbreviations: paO$_2$, partial oxygen pressure; paCO$_2$, partial carbon dioxide pressure.
8.3.2 Total NOS activity

Total NOS activity measured at day 4 after induced MCAO was shown in Figure 8.2. Statistical analysis indicated that NOS activity in each treatment group was significantly different from the Normal group (290.43 ± 43.16 pmol nitrate produced per hr per mg protein) (P<0.05), suggesting that the ischemia-reperfusion could induce NOS activity [Fassbender et al., 2000]. The total NOS activity was maintained in the Control groups after 3-times and 18-times EA stimulation. In comparison to the Control group, the NA groups possessed about 15% increased total NOS activity in both of the 3-times and 18-times EA stimulation (P<0.05). However, an upregulated total NOS activity was observed in the GB20 or ST36 group after 18-times EA stimulation. The mean value of total NOS activity in the ST36 group (472.09 ± 21.47 pmol nitrate produced/hr/mg protein) was comparable to that of the NA group at the same treatment. But the enzymatic activity in the GB20 group was increased to 655.33 pmol nitrate produced/hr/mg protein and was significantly higher than those of the Control group and ST36 group (P<0.05). In addition, by comparing the total NOS activity at two treatment times, except the Control groups, all the remaining three groups exhibited further induced NOS activity at 18-times EA stimulation (P<0.05). One-tenth increase in enzymatic activity were observed in the NA and ST36 groups, while over 60% increase was found in the GB20 groups.

8.3.3 Expression of NOS isoforms

Western blotting analysis of brain homogenates using antibodies specifically directed nNOS, iNOS and eNOS obtained was shown in Figure 8.3A, 8.4A, and 8.5A, respectively. Amount of them quantified by densitometer was shown in Figure 8.3B, 8.4B and 8.5B, accordingly. β-actin was used as the internal control. In Figure 8.3,
the amount of nNOS expressed in each experimental group was significantly greater than that of the Normal group (222.29 ± 7.03 pg nNOS per mg protein) (P<0.05). In the 3-times EA stimulation, there was no difference between the Control group, NA group and GB20 group. The ST36 group produced a significantly greater amount of nNOS than other experimental three groups did (P<0.05). In the 18-times EA stimulation, the nNOS expression was greatly increased to 377.46 ± 52.67, 555.23 ± 67.13, and 462.61 ± 50.08 pg nNOS per mg protein, respectively, in the NA group, GB20 group and ST36 group. These values were found to be significant from that of the Control group (P<0.05). By comparing the expression values of the same group at two treatment times, the 18-times EA could induce more nNOS expression in all the experimental groups (P<0.05), except the Control group.

As shown in Figure 8.4, except the GB20 group at 3-times EA stimulation, iNOS expression of all the remaining groups were significantly greater than that of the Normal group (P<0.05). In the 3-times EA stimulation, the iNOS expression was found to be the least in the GB20 group (about 93 pg iNOS per mg homogenate), but it was only significantly differed from the NA group. After the 18-times EA stimulation, in comparison to the Control group, the mean value of iNOS expression was further increased by 16% in the NA group and by 30% in the GB20 group (P<0.05). Such a significant increase was not observed in the ST36 group. Moreover, there was a remarkably difference of iNOS expression between the GB20 group and the ST36 group (P<0.05). Statistical comparison of iNOS expression of the same group with different treatment times found that only the GB20 group and ST36 group was further increased after 18-times EA stimulation.
Figure 8.2. Total nitric oxide synthase (NOS) activity of the normal rats and ischemic rats with multiple pre-ischemic EA stimulation. Data (mean ± standard deviation) obtained from the right brain of rats 4 days after MCAO in each experimental group (n=6), except in the Normal group (n=8). Total NOS activity is defined as pmol nitrate produced per hour per mg protein. Statistical analysis reveals that there is a significant difference between the Normal group and each experimental group (P<0.05). Under the same treatment times, symbols (b,c,d) indicate the significance differences in comparison to the Control group (P<0.05) and to the NA group (P<0.05), and between the GB20 group and ST36 group (P<0.05), respectively. Symbol (e) represents the significant difference by comparing the group at 18-times EA stimulation to its corresponding group at 3-times EA stimulation.
Figure 8.3. Expression of neuronal nitric oxide synthase (nNOS): (A) Selected western blot specific for nNOS of the experimental groups with β-actin as internal control. (B) Quantification of nNOS detected (mean ± standard deviation) in each experimental group (n=6) and in the Normal group (n=8). Unit is defined as pg nNOS expressed per mg protein. Statistical analysis reveals that there is a significant difference between the Normal group and each experimental group (P<0.05). Under the same treatment times, symbols (b,c,d) indicate the significance differences in comparison to the Control group (P<0.05) and to the NA group (P<0.05), and between the GB20 group and ST36 group (P<0.05), respectively. Symbol (e) represents the significant difference by comparing the group at 18-times EA stimulation to its corresponding group at 3-times EA stimulation.
Figure 8.4. Expression of inducible nitric oxide synthase (iNOS): (A) Selected western blot specific for iNOS of the experimental groups with β-actin as internal control. (B) Quantification of iNOS detected (mean ± standard deviation) in each experimental group (n=6) and in the Normal group (n=8). Unit is defined as pg iNOS expressed per mg protein. Statistical analysis reveals that, except the GB20 group at 3-times EA stimulation, there is a significant difference between the Normal group and each experimental group (P<0.05). Under the same treatment times, symbols (b,c,d) indicate the significance differences in comparison to the Control group (P<0.05) and to the NA group (P<0.05), and between the GB20 group and ST36 group (P<0.05), respectively. Symbol (e) represents the significant difference by comparing the group at 18-times EA stimulation to its corresponding group at 3-times EA stimulation.
According to the Figure 8.5, only the experimental groups after 18-times EA stimulation produced a significant increase in eNOS expression in comparison to the Normal group (P<0.05). All the experimental groups after 3-times EA stimulation expressed similar levels of eNOS which were comparable to that of the Normal group. In the 18-times EA stimulation, the GB 20 group produced the highest amount of eNOS (189.14 ± 41.07 pg eNOS per mg protein) which was significant to all the three groups (P<0.05). Unlike nNOS and iNOS, the associated increase of eNOS with increased treatment times was only observed in the GB20 and ST36 group (P<0.05).

8.3.4 Expression of transforming growth factor β-1 (TGFβ-1) and Bel-2

Referring to Fig. 8.6, TGFβ-1 was not detected in the Normal group. After 3-times EA stimulation, no difference of expression was observed among the Control group, NA group and GB20 group. However, the expression in the ST36 group was significantly increased to 4.44 ± 1.18 pg TGFβ-1 per mg protein (P<0.05). Similarly, the ST36 group after 18-times EA stimulation expressed the greatest amount of TGFβ-1 (6.72 ± 1.99 pg TGFβ-1 per mg protein) that was significantly higher than those of other three groups. Additionally, the GB20 group at the same treatment times also produced two-fold more TGFβ-1 than the Control group did (P<0.05). Nevertheless, the associated increase in expression with the increase in treatment times was demonstrated in ST36 groups in which the 18-times EA stimulation gave about 50% increase (P<0.05).
### Figure 8.5. Expression of endothelial nitric oxide synthase (eNOS): (A) Selected western blot specific for eNOS of the experimental groups with β-actin as internal control. (B) Quantification of eNOS detected (mean ± standard deviation) in each experimental group (n=6) and in the Normal group (n=8). Unit is defined as pg eNOS expressed per mg protein. Symbol (a) represents a significant difference to the Normal group (P<0.05). At the 18-times EA stimulation, symbols (b,c,d) indicate the significance differences in comparison to the Control group (P<0.05) and to the NA group (P<0.05), and between the GB20 group and ST36 group (P<0.05), respectively. Symbol (e) represents the significant difference by comparing the group at 18-times EA stimulation to its corresponding group at 3-times EA stimulation.
Figure 8.6. Expression of transforming growth factor beta-1 (TGFβ-1): (A) Selected western blot specific for TGFβ-1 of the experimental groups with β-actin as internal control. (B) Quantification of TGFβ-1 detected (mean ± standard deviation) in each experimental group (n=6) and in the Normal group (n=8). Unit is defined as pg TGFβ-1 expressed per mg protein. Statistical analysis reveals that there is a significant difference between the Normal group and each experimental group (P<0.05). Under the same treatment times, symbols (b,c,d) indicate the significance differences in comparison to the Control group (P<0.05) and to the NA group (P<0.05), and between the GB20 group and ST36 group (P<0.05), respectively. Symbol (e) represents the significant difference by comparing the group at 18-times EA stimulation to its corresponding group at 3-times EA stimulation.
In Figure 8.7, the amount of Bcl-2 expressed in the Normal group was $0.502 \pm 0.06$ pg Bcl-2 per mg protein. In the 3-times EA stimulation, a significant increase in Bcl-2 expression was only observed in ST60 group ($P<0.05$). In the 9-times EA stimulation, the Bcl-2 expression was upregulated in all the groups as compared to those in the 3-times EA stimulation. Again, in comparison to the Control group and GB20 group, the ST36 group was found to express greater Bcl-2 by 29% and 15%, respectively ($P<0.05$). However, the Bcl-2 expression was also upregulated in NA group that was not differed from ST36 group.

### 8.3.5 Lipid peroxidation

Changes in MDA concentration in the rat right brain after multiple pre-ischemia EA stimulation were shown in Figure 8.8. Only the Control group in the 3-times EA stimulation and NA groups at both frequencies of EA stimulation produced more than two-times of MDA concentration ($P<0.05$) than the Normal group ($4.87 \pm 0.44$ µM per mg protein). On the other hand, significant difference was observed when comparing concentrations of MDA between groups of each treatment frequency. In the 3-times EA stimulation before cerebral ischemia, the NA groups showed a remarkably increased MDA amount in comparison to the Control group ($p<0.05$). Furthermore, its concentration is four-times that of the GB20 group and three-times that of the ST36 group ($p<0.05$). Only the GB20 group but not the ST36 group, produced nearly 40% of the concentration of MDA of the Control group ($p<0.05$). Similarly, the NA group in the 18-times EA stimulation had the highest MDA amount detected, which was two-fold greater than those of GB20 and ST36 groups. It may imply that the use of this non-acupoint would exacerbate the lipid peroxidation,
suggesting the significant relationship between acupoints and meridians. Although
the GB20 and the ST36 groups produced lesser MDA amount than that of the Control
group (p<0.05), there was no significant difference between them. These may suggest
that both the GB20 and ST36 groups could effectively regulate the MDA production.
Comparison of MDA amounts of the same groups with two different frequencies of
EA therapy found that there was no significant difference. This suggested that the
increased frequency of EA stimulation before cerebral ischemia was not related to the
decreased MDA production.

Changes in total concentration of MDA and 4-HNE in the rat right brain after multiple
pre-ischemia EA stimulation were shown in Figure 8.9. The basal value of MDA and
4-HNE obtained from the Normal group was 21.38 ± 1.05 µM per mg protein. After
pre-ischemic EA stimulation for either 3 or 18 times, only the NA groups produced
greater concentrations of MDA and 4-HNE than the Normal group did. In the 3-times
EA stimulation before cerebral ischemia, the NA groups showed a remarkably
increased amount of MDA and 4-HNE when comparing to the GB20 and ST36
groups (p<0.01). The mean value of GB20 and ST36 groups was only 55% of that of
the NA group. However, this increase was not observed when the 18-times EA
stimulation was used. In addition, the amount of MDA and 4-HNE of the each group
with different number of EA stimulation was not found to be statistically significant
(p>0.05). It suggested that the number of EA therapy was not critical to attenuate the
lipid peroxidation.
Figure 8.7. Expression of Bcl-2: (A) Selected western blot specific for Bcl-2 of the experimental groups with β-actin as internal control. (B) Quantification of Bcl-2 detected (mean ± standard deviation) in each experimental group (n=6) and in the Normal group (n=8). Unit is defined as pg Bcl-2 expressed per mg protein. Statistical analysis reveals that there is a significant difference between the Normal group and each experimental group (P<0.05). Under the same treatment times, symbols (b,c,d) indicate the significance differences in comparison to the Control group (P<0.05) and to the NA group (P<0.05), and between the GB20 group and ST36 group (P<0.05), respectively. Symbol (e) represents the significant difference by comparing the group at 18-times EA stimulation to its corresponding group at 3-times EA stimulation.
Figure 8.8. Changes in concentration of MDA of different groups after multiple pre-ischemia EA stimulation. Data (mean ± standard deviation) obtained from the right brain of rats 4 days after MCAO in each group (n=6), except in the Normal group (n=4). The concentration is defined as [MDA] (µM) per mg homogenate protein. Symbols (a, b, c) represent the significance of difference to the Normal group (P<0.05), to the Control group (P<0.05) and to the NA group (P<0.05) at corresponding number of EA stimulation, respectively.
Figure 8.9. Changes in total concentration of MDA and 4-HNE of different groups after multiple pre-ischemia EA stimulation. Data (mean ± standard deviation) obtained from the right brain of rats 4 days after MCAO in each group (n=6), except in the Normal group (n=4). The unit is defined as [MDA + 4-HNE] (µM) per mg protein. Symbols (a, c) represent the significance of difference to the Normal group (P<0.05), and to the NA group (P<0.05) at corresponding number of EA stimulation, respectively.
8.4 DISCUSSION

The intraperitoneal injection of ketamine-xylazine could attenuate the lipopolysaccharide-induced expression of iNOS in rat gastric mucosa [Helmer et al., 2003a], liver [Helmer et al., 2003b] and alveolar macrophages [Li et al., 1997]. This study has first examined if the multiple use of anesthetic drugs could alter the enzymatic activities and protein expression in brain tissues. Taking the total NOS activity \textit{ex vivo} into consideration, it remained unchanged in two Control groups with ketamine-xylazine anesthesia for 3 or 18 times prior to induction of cerebral ischemia. Referring to the protein expressions, especially the iNOS expression, there was no observable difference in two Control groups. The observations suggested that the multiple uses of ketamine and xylazine would produce neither the neuroprotective nor neurotoxic effect on brain tissue that was in line with the observations from other previous studies [Dawson et al., 1971; Garcia, 1984; Molinari and Laurent, 1976; Takeshita et al., 1972; Wu et al., 2000; Yang et al., 2002]. They found that ketamine, in contrast to the neuroprotective effect by barbiturates [Hoff et al., 1975; Smith et al., 1974; Yatsu et al., 1972], would exert a neutral effect on brain tissues [Garcia, 1984; Molinari and Laurent, 1976; Takeshita et al., 1972]. Wu et al. (2000) also demonstrated that NO concentration in the rat hippocampus and striatum had no direct relationship to anaesthesia induced by intraperitoneal injection of 100 mg/kg ketamine. In this study, therefore, the changes found in total NOS activity or its protein expression was not related to the multiple uses of ketamine and xylazine.

Our results showed that the induction of cerebral ischemia could significantly induce the NOS activity and its protein expression that was previously reported in other studies. NO production was found to be greatly increased in day 2 or day 3 after
transient focal ischemia [Fassbender et al., 2000; Grandati et al., 1997], which was mainly contributed by the up-regulated iNOS expression [Grandati et al., 1997, Iadecola et al., 1995; Loreouet et al., 2002]. Up-regulation of nNOS and eNOS was also found to induce the NO production [Leker et al., 2001; Zhang et al., 1993]. Increased expression of nNOS and iNOS are thought to mediate the neuronal injury [Samdani et al., 1997; Wei et al., 1999], whereas the increased expression of eNOS is thought to mediate changes in cerebral blood flow [Zhang et al., 1993]. Nevertheless, when a huge amount of NO was produced by nNOS and iNOS, it was impossible to significantly remove it by the upregulated eNOS level [Leker et al., 2001]. Induction of NO production would lead to the increase in levels of lipid peroxidation, which was assessed by the amount of MDA and 4-HNE that are cytotoxic to neuronal cells. 4-HNE exhibited a wider specific reactivity on DNA and membrane, while MDA only had specificity on proteins. However, the production of MDA was more rapidly and dominantly induced in different brain regions and plasma under the ischemia-reperfusion model. For examples, in the four-vessel occlusion model, MDA level was not increased until recirculation started [Bromont et al., 1989]. During the first hour of recirculation, MDA levels were increased in the cortex, hippocampus, and striatum. Before 72 hours of recirculation, a substantially increasing amount of MDA was observed only in cortex. After 72 hours, the MDA level in all regions increased with the time of recirculation. Similarly, plasma samples from stroke patients were detected with an increased level of MDA but not the total level of MDA and 4-HNE [Re et al., 1997]. Up-regulated MDA level was likely as a result of increased activities of neutrophils in inflammation caused by cerebral ischemia [Re et al., 1997]. Therefore, it is generally believed that any therapy to ameliorate the NO-mediated
Application of EA stimulation after cerebral ischemia had been reported to reduce NO production and inhibit NOS activity in brain tissues of rats [Xu et al., 1996; Zhao et al., 2000] and plasma of stroke patients [Wang et al., 2001]. In contrast, no inhibitory effect was observed in this study where EA stimulation was applied prior to cerebral ischemia. Firstly, the results found that total NOS activity was further increased after multiple application of pre-ischemia EA stimulation. Secondly, such an increase was parallel with an up-regulated expression of each type of NOS. Thirdly, the increased activity and upregulated expression was directly related to the number of pre-ischemia EA stimulation. In comparison to the Control group (rats with ischemia only), the total NOS activities in all the treatment groups after 3-times EA stimulation were not altered while those after 18-times EA stimulation were greatly increased. As a consequence, the level of lipid peroxidation induced by NO-mediated toxicity is expected to be higher. However, our previous studies found that the MDA and 4-HNE levels were maintained at the level comparable to the Control groups after multiple pre-ischemic EA stimulation. In this regard, there may possibly be another cellular protective system induced after a huge amount of NO production that could encounter the effect of the NO-mediated toxicity, thereby resulting in decreased level of lipid peroxidation.

The expression of TGFβ-1 was examined as it was a delayed response gene that was suitable for the time of harvest in our study. Its expression level was persistently maintained from 2 to 15 days after cerebral ischemia [Wang et al., 1995; Yamashita et
al., 1999]. It was also reported that TGFβ-1 expression reached the maximum at day 4 and declined at day 7 after cerebral ischemia [Ata et al., 1999; Knuckey et al., 1996; Lehrmann et al., 1995]. In our study, the induction of cerebral ischemia led to the upregulation of TGFβ-1 expression. No TGFβ-1 protein was detected in the intact brain tissue that was in line with other studies the TGFβ-1 expression was minimal in normal brain tissue [Henrich-Noack et al., 1996]. But it was significantly produced in ischemic rats [Henrich-Noack et al., 1996]. In this study, rats with EA stimulation at GB20 or ST36 were found to have a further increase in TGFβ-1 expression. The enhanced expression of TGFβ-1 serves several effects. For examples, TGFβ-1 could reduce NO production by reducing expression of iNOS [Iadecola et al., 1995; Minc-Golomb et al., 1994]. Both in vitro administration of TGFβ-1 and in vivo expression of TGFβ-1 suppressed the expression of nNOS [Henrich-Noack et al., 1996; Lehrmann et al., 1995] and iNOS [Gross et al., 1993; Gross et al., 1994; Perrella et al., 1996] and thereby reduce the infarct size. Moreover, the increased TGFβ-1 levels could induce the eNOS expression [Inoue et al., 1995; Lefer et al., 1993] that could produce the vasodilating effect, hence reducing the infarct size. In addition, it could upregulate the expression of brain-derived neurotrophic factor (BDNF) [Zhu et al., 2001] and inhibit the apoptotic cascade induced by NO via increasing Bcl-2 protein expression which was also found to be induced in this study [Flanders et al., 1998; Prehn, et al., 1994; Zhu et al., 2001]. Therefore, the induced level of TGFβ-1 was likely producing the possible protection against the NO-mediated neurotoxicity, thereby suppressing lipid peroxidation and inhibiting the apoptotic events.

This study has compared the effect of EA stimulation at GB20 and ST36 on regulating the enzymatic activity, protein expression, and lipid peroxidation. Our
results showed that EA stimulation at either acupoint could not suppress the NOS activity or expression, but increase the TGFβ-1 expression. In consequence, they could reduce the level of MDA production and restore it to the normal level. Either at the 3-times or 18-times EA stimulation, the lowest amounts of MDA were observed in the GB20 groups, which were half of the values in the Control group (P<0.05). This significant reduction was only observed in the ST36 group after 3-times pre-ischemic EA stimulation. Our results are in line with the reduced cerebral infarction, which was as a result of lipid peroxidation, after EA stimulation at either GB20 or ST36 [Chen and Fang, 1990; Zou and Wang, 1990]. In the case of the functional abilities, such as locomotion [Liu et al., 1999] and speech ability [Zhang and Ni, 1998], stroke patients with EA stimulation at GB20 on stroke patients had a greater extent of recovery than those with EA stimulation at ST36. However, our statistical analysis revealed that there was no observable difference of MDA amount between the GB20 and ST36 group. It may imply that both EA stimulation at GB20 or ST36 could produce a comparable and beneficial effect at the cellular level. On the other hand, at the cellular level, EA stimulation at ST36 could effectively induce the TGFβ-1 expression that increases the expression of brain-derived neurotrophic factor (BDNF) as previously reported [Yun et al., 2002; Zhu et al., 2001]. Therefore, it was impossible to differentiate which acupoint was better for use in pre-ischemia treatment.

In addition, the significance of the selection of appropriate acupoints was demonstrated by the NA groups (rats with EA stimulation at non-acupoints). The non-acupoints were bilaterally located near to a pair of well-known acupoint, Huantiao (GB30). Huantiao is located on the outer 1/3 distance between the tip of
cocyx and hip joints [Ellis et al., 1991]. Previous studies found that the amount of MDA in the ischemic brain of adult rats was decreased after the EA stimulation on Huantiao [Chen et al., 1998]. However, total amount of MDA and 4-HNE produced as well as the amount of MDA alone were found to be the highest in the NA groups. It was about 30% more than the values found in the Control group (P<0.05). These results implied that the non-acupoint used was highly unlikely to be Huantiao. Although EA stimulation at non-acupoint could produce lesser induction of NOS activity and expression, it could not effectively induce more TGFβ-1 expression that produced the neuroprotection in brain tissues. It further suggested that providing EA stimulation at non-acupoints may be detrimental to the health of stroke patients.

Nevertheless, when measuring both MDA and 4-HNE, the EA stimulation at neither GB20 nor ST36 could reduce their amounts as comparable as the Control group. This failure was unlikely associated with the reliability of the assay method. The used method showed much less interference than that by the determination of thiobarbituric acid reaction substance (TBARS) previously used [Erdelmeier et al., 1998; Esterbauer, 1996]. The failure may be partly related to the time of measurement. Kondo et al. (1997) found that level of MDA and 4-HNE would only be enhanced in the frontal cortex and hippocampus within 1 day after transient focal cerebral ischemia. Except the striatum, none of the brain regions were found to increase the total amount of MDA and 4-HNE during 1 week to 24 weeks after ischemia. It may suggest that harvest at post-ischemia day 4 was not an appropriate time for the measurement of total MDA and 4-HNE. Moreover, the number of animals used may also contribute to the failure. Statistical analysis revealed that the observed power of this measurement was only 0.481, whereas the observed power of measurement of MDA only was 0.961.
These findings suggested that more animals should be recruited for the measurement of total lipid peroxidation products.

Unlike previous studies that focused on the stroke rehabilitation by EA stimulation, this study could provide another view of clinical application of EA stimulation. The pre-ischemic EA stimulation is particularly important to those high-risk groups such as patients with high blood pressure. Keeping EA stimulation at either GB20 or ST36 at alternative days per week may ameliorate the severity of brain damage after stroke attack, so as to shorten the time of the recovery.

8.5 CONCLUSION

To conclude, multiple pre-injury EA stimulation at either GB20 or ST36 can effectively increase the TGFβ-1 and Bcl-2 expression and reduce the amount of MDA resulted from lipid peroxidation induced by cerebral ischemia-reperfusion. According to the specific reactivity of MDA, reducing MDA amount by EA stimulation may promote the protection of protein against oxidative stress. In addition, the TGFβ-1 could exert the neuroprotection by regulating the apoptotic cascade. Owing to the limited sample size, this beneficial effect was not truly revealed in measuring the total amount of MDA and 4-HNE. Further experiment recruiting a large animal size for the measurement of total MDA and 4-HNE and for evaluation of apoptotic events is suggested.
9.1 ORIGINAlITY OF THE STUDY

In the present study, two acupoints were selected, Fengchi (GB20) and Zusanli (ST36), which are commonly used for stroke rehabilitation. Beneficial effects have been reported when using EA stimulation at these acupoints, including an improved speech ability [Liu et al., 2000; Zhang and Ni, 1998; Zhao et al., 1998], improved locomotion [Liu et al., 1999; Tan, 1990; Zhang and Ni, 1998], and enhanced memory [Li and Shen, 1998]. However, it still remains unclear how the EA stimulation could improve the outcomes and disability of patients suffered from stroke. Therefore, the ultimate aim of the research study is to investigate the effects of the post-ischemia electroacupuncture (EA) stimulation on regulating the reactive oxygen species-mediated neuronal injury and the apoptotic neuronal damage. To my best of knowledge, the present study provides several novel findings and observations. Firstly, the EA stimulation at either GB20 or ST36 seemed to reduce the lipid peroxidation by modifying the activity and expression of nitric oxide synthase (NOS) as well as by increasing the coupled enzyme activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Chapter 4). Secondly, the EA stimulation was noted in preventing protein oxidation induced by reactive oxygen species (ROS) through the enhanced expression of thioredoxin (Trx) and stimulated activity of thioredoxin reductase (TR) (Chapter 5). Thirdly, the EA stimulation was able to suppress the initiation of apoptotic events by both suppressing the activation of caspases (caspase-9 and caspase-3) and the dephosphorylation of apoptotic proteins.
(BAD), as well as by triggering the expression of anti-apoptotic proteins (TGF\(\beta\)-1, Bcl-2 and phosphorylated Akt) (Chapter 6). Finally, the aforementioned effectiveness of EA stimulation was maintained by applying the EA stimulation several days after cerebral ischemia (Chapter 7) or applying multiple sessions of EA stimulation prior to cerebral ischemia (Chapter 8). Taken together, the new findings suggested that EA stimulation could improve the cellular damage induced by cerebral ischemia-reperfusion, and therefore, it may be useful to explain the beneficial effects observed clinically.

### 9.2 SUMMARY OF FINDINGS

Effects of using a single application of post-ischemic EA stimulation at either Fengchi (GB20) or Zusanli (ST36) were tabulated in Table 9.1 that summarized the results obtained from Chapter 4 to Chapter 6. A schematic diagram illustrating the findings and the relationship of these parameters was constructed in Figure 9.1. Each of the relationship labelled in a numeric order is discussed in the following:

1. Results showed that the thioredoxin (Trx) expression was significantly increased in day 4 after the transient focal cerebral ischemia and EA stimulation (Figure 5.3). The increased Trx would likely induce the expression of manganese superoxide dismutase (MnSOD) as observed in Figure 4.7 [Das et al., 1997; Rabilloud et al., 2001; Tanaka et al., 2002]. This can facilitate the removal of the superoxide anions generated from the mitochondrial leakage induced by cerebral ischemia-reperfusion injury. Higher removal rate of superoxide anions can effectively protect brain cells against nitric oxide-induced injury [Brockhaus and Brune, 1999; Das et al., 1997; Gonzalez-Zulueta et al., 1998; Huie and Padmaja, 1993].
Table 9.1 Summary of findings of the use of post-ischemia EA stimulation on rats with the cerebral ischemia-reperfusion injury.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>EA stimulation at GB20</th>
<th>EA stimulation at ST36</th>
<th>Figure</th>
</tr>
</thead>
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<tr>
<td><strong>Chapter 4</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NOS activity</td>
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<td>↓d4 b,c</td>
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</tr>
<tr>
<td>nNOS expression</td>
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<td>↓d4 b,c</td>
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<td>iNOS expression</td>
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<td>↓d1,d4 b,c</td>
<td>4.3</td>
</tr>
<tr>
<td>eNOS expression</td>
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<td>↓d4 b,c</td>
<td>4.4</td>
</tr>
<tr>
<td>Total SOD activity</td>
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<td>↑d1,d4 a,b,c</td>
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<td>MnSOD expression</td>
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<td>↑d1,d4 a,b,c</td>
<td>4.7</td>
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<td>GPx activity</td>
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<td>↑d1,d4 a,b,c</td>
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<td>↓d1,d4 b,c</td>
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<td>MDA + 4-HNE levels</td>
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<tr>
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<td>No</td>
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</tr>
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<td>reduction</td>
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<tr>
<td>TR activity on IgG</td>
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<tr>
<td>Trx expression</td>
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</tr>
<tr>
<td>pAkt expression</td>
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<td>↑d1,d4 a</td>
<td>6.2</td>
</tr>
<tr>
<td>BAD expression</td>
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<td>No</td>
<td>6.3</td>
</tr>
<tr>
<td>pBAD expression</td>
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<td>↑d1,d4 a,b</td>
<td>6.8</td>
</tr>
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</table>

Symbols (a, b, c) denote the significant differences in comparison to the normal rats, to the rats with ischemia only, and to the rats with Sham EA stimulation at the corresponding acupoints, respectively. Symbols (↑ and ↓) imply the increase and decrease of expression or activity measured, respectively. Abbreviations (d1 and d4) represent the day 1 and day 4 of cerebral ischemia-reperfusion injury. Word (No) implies no altered effect observed in comparison to the normal rats and rats with ischemia induction.
Figure 9.1 Summary of the effect and relationship of the parameters measured in the research study.
2. Increased Trx can regenerate the reduced form of glutathione (GSH) which is necessary to maintain the thiol-redox balance within the cell. Under cerebral ischemia, the reactive nitric oxide readily reacted with GSH to form S-nitrosoglutathione (GSNO) [Burton, 1985; Gaston et al., 1993]. The depleting level of GSH in turn limited the activities of glutathione peroxidase (GPx) [Luthman and Holmgren, 1982; Takagi et al., 1998], leading to reduced ability on removing ROS intermediates. In our study, over-expression of Trx by EA stimulation is likely to restore the active GSH by reducing the GSNO formed [Burton, 1985; Gaston et al., 1993] and therefore re-activate the GPx activity. In addition, Trx could act as the substrate for GPx [Arnér and Holmgren, 2000; Gromer et al., 1999; Lippoldt et al., 1985; Mustacich and Powis, 2000; Nakamura et al., 1994; Poole et al., 2000; Takagi et al., 1998]. Dual actions of Trx probably ensured that GPx was fully active in protecting the cells from the injury by hydrogen peroxide (Figure 4.8).

3. The increased Trx expression associated with the upregulated MnSOD expression may lead to the suppression of caspase-3 as observed in Figure 6.6 [Baker et al., 2000; Das et al., 1997; Noshita et al., 2001; Rabilloud et al., 2001; Tanaka et al., 2002] and thereby inhibiting the ultimate DNA fragmentation.

4. Thioredoxin reductase (TR) activity was increased at four days after focal cerebral ischemia and EA stimulation at ST36 (Figure 5.2). This increased activity may incorporate with the induced Trx expression to protect the cells by efficient scavenging of reactive oxygen intermediates, such as hydrogen peroxide and hydroxyl radicals [Arnér and Holmgren, 2000; Gromer et al., 1999; Mustacich
and Powis, 2000; Nakamura et al., 1994; Poole et al., 2000; Takagi et al., 1998].

5. Our results found that nNOS expression as well as the total NOS activity were reduced (Figure 4.1). Observations were in line with the observation that EA stimulation could reduce the NO production [Zhao et al., 2000]. The decreased NOS activity could possibly be due to the suppressed release of excitatory amino acids that triggered the influx of extracellular calcium [Zhao et al., 1997]. It therefore inhibited the calcium-dependent nNOS activity that contributed to the total NOS activity. It would further lead to the reduced amount of iNOS through the associated down-regulation of the transcription factor nuclear factor kappa-B (NFκB) [Togashi et al., 1997]. On the other hand, EA stimulation had been found to down-regulate the mRNA and protein expression of interleukin-1 (IL-1) [Xu et al., 2002; Zhang et al., 1994]. It can suppress the IL-1-dependent induction of iNOS expression. As a consequence, the decreased nNOS and iNOS protein could decrease the total NOS activity and hence reduce the cerebral infarct volume [Fassbender et al., 2000; Nagafuji et al., 1995b; Yoshida et al., 1994; Zhang et al., 1996].

6. The results showed that transforming growth factor β-1 (TGFβ-1) expression was remarkably induced at day 1 and day 4 after cerebral ischemia and EA stimulation (Figure 6.8). Being a delayed response gene [Ata et al., 1999; Knuckey et al., 1996; Lehrmann et al., 1995; Wang et al., 1995; Yamashita et al., 1999], enhancing its expression as early as 1 day after EA stimulation may highly potentiate the neuroprotection against the NO-mediated toxicity and apoptosis. TGFβ-1 contributes to the calcium homeostasis in nerve cells that is important to
regulate the expression of nNOS [Henrich-Noack et al., 1996; Lehrmann et al., 1995] and the iNOS expression [Gross et al., 1993; Gross et al., 1994; Iadecola et al., 1995; Minc-Golomb et al., 1994; Perrella et al., 1996; Thomas et al., 2001]. In this study, an increased expression of TGFβ-1 would likely reduce NO production by suppressing the expression of both nNOS and iNOS as shown in Figure 4.2 and Figure 4.3.

7. Another beneficial effect of TGFβ-1 was noted to effectively increase the phosphorylation of BAD (Bcl-2-associated death protein) as observed in Figure 6.4 [Zhu et al., 2002] or to avoid the phosphorylated BAD (pBAD) from dephosphorylation [Richman et al., 1999]. It could then result in reduced apoptotic bodies observed in brain tissues [Horowitz et al., 2004; Zhu et al., 2002].

8. The increased expression TGFβ-1 could also induce the expression of Bcl-2 as observed in Figure 6.7 [Flanders et al., 1998; Prehn et al., 1994; Zhu et al., 2001]. Increased expression of Bcl-2, either by over-expression of itself or the use of antisense treatment, could suppress the release of cytochrome c and activation of pro-caspase 9 [Seo et al., 2002; Shimizu et al., 2001; Zhao et al., 2003], resulting in attenuated infarct volume.

9. In addition to induction by TGFβ-1, it has been reported that Bcl-2 was co-expressed with the 4-hydroxynonenal-modified proteins in microglial cells and neurons of rat brain after transient focal ischemia [Urabe et al., 1998]. It suggested that Bcl-2 may play an important role in promoting the survival of neurons and activated microglia [Urabe et al., 1998]. Therefore, the rise in MDA
and 4-HNE amount by transient focal ischemia (Figure 4.9) may further induce
the Bcl-2 expression. Moreover, the increased Bcl-2 protein may be associated
with the increased levels of basic fibroblast growth factor (bFGF) [Ay et al., 2001]
in striatum and cortex which could be induced by EA stimulation [Ou et al., 2001].

10. Over-expression of the CuZnSOD could activate the serine/threonine kinase (Akt)
by phosphorylation at serine-473 (ser-473) and then initiate the activation of the
Akt-cell-survival signaling pathway and attenuate the subsequent DNA damage
[Noshita et al., 2003]. However, unlike the induced expression level of MnSOD,
the expression levels of copper-zinc superoxide dismutase (CuZnSOD) were
decreased (Figure 4.5). The decreased CuZnSOD expression was not in line with
the upregulation of pAkt expression as observed in Figure 6.2. On the other hand,
the upregulated pAkt expression was likely related to the effect of TGFβ-1, which
could induce the activation of Akt [Chen et al., 2001; Horowitz et al., 2004].

11. Phosphorylated Akt (pAkt) could activate the endothelial nitric oxide synthase
(eNOS) by phosphorylating the serine-1177 (ser-1177) in eNOS [Boo et al., 2002;
Michell et al., 1999; Papapetropoulous et al., 2004]. This activation was not
observed when the specific inhibitor of pAkt, wortmannin, was used [Dimmeler et
al., 1999] or when the mutant eNOS (S1179A) was used [Fulton et al., 1999].
Therefore, the diminished levels of eNOS (Figure 4.4) may not be related to the
pAkt expression which was upregulated in this study. On the other hand, the
reduced eNOS expression may be related to the decreased expression of
CuZnSOD that induced the eNOS expression and protected eNOS from protein
nitration due to the vigorous NO production by nNOS and iNOS [Brennan et al.,
12. As a whole, the MDA and 4-HNE levels were reduced in this study (Figure 4.9). It was as partly resulted from the concomitant increase of SOD and GPx activity that can effectively remove the superoxide anion and the subsequent hydrogen peroxide [Crack et al., 2003; Trépanier et al., 1996; Weisbrot-Lefkowitz et al., 1998]. The reduced levels of nNOS and iNOS may also contribute to the decreased levels of lipid peroxidation [Fassbender et al., 2000; Nagafuji et al., 1995b; Yoshida et al., 1994; Zhang et al., 1996]. At the same time, the simultaneously decreased availability of nitric oxide and superoxide anion would protect the brain tissues from the formation of peroxynitrite which is highly reactive to the lipid membrane [Gonzalez-Zulueta et al., 1998; Huie and Padmaja, 1993; Kumura et al., 1996; Watson et al., 1996].

13. On the other hand, the decreased levels of caspase-9 and the following caspase-3 activity were found in this study (Figure 6.5 and Figure 6.6). Inhibition of the caspase-9 [Cao et al., 2002; Mouw et al., 2002] and caspase-3 [Bilsland and Harper, 2002; Davoli et al., 2002; Gill et al., 2002; Love et al., 2000] could improve the apoptotic injury induced by reversible focal ischemia in rats. The decreased levels were likely as a result of the action by Bcl-2 that could suppress the release of cytochrome c from the mitochondria [Shimizu et al., 2001; Zhao et al., 2003]. The increased phosphorylated BAD levels may also keep away the dephosphorylated BAD protein from forming dimer with the anti-apoptotic Bcl-
XL protein (Bcl-associated XL protein) and then displacing the BAX protein (Bcl-associated X protein) that translocates to the mitochondria and stimulates the release of cytochrome c [Graham and Chen, 2001; Henshall et al., 2002]. However, the suppression of BAD was partly contributed by the phosphorylated Akt because the pAkt levels were unregulated [Ouyang et al., 1999; Saito et al., 2003].

9.3 COMPARISON OF THE EFFECTS OF EA STIMULATION AT FENGCHI (GB20) AND ZUSANLI (ST36)

As mentioned before, two acupoints Fengchi (GB20) and Zusanli (ST36) were used for comparison in this study. However, as a whole, the effects of EA stimulation at GB20 and ST36 were found to be comparable to each other. It may help explaining the comparable effects as observed in clinical situation in relation to stroke (as summarized in Table 9.2): Although they share similar and comparable clinical outcomes, GB20 is more commonly used for patients with stroke. Previous studies using EA stimulation at a pair of bilateral GB20 acupoints reported that there were some beneficial effect were found, such as a better regulation of cerebral blood flow rate [Cui, 1994; Liu et al., 2000; Yuan et al., 1996; Yuan et al., 1998], the reduction of the blood lipid levels [Zhou et al., 1995], the decreased level of acute fever [Huang, 1994; Tan, 1992] and the increased activity of superoxide dismutase and the corresponding decreased levels of lipid peroxidation in plasma [Lai et al., 1997]. Moreover, some clinical studies further compared the effectiveness of the use of EA stimulation at GB20 and the use of EA stimulation at ST36. It was found that the stroke patients with EA stimulation at GB20 had an increased ability of locomotion.
[Liu et al., 1999] and the improved speech ability [Guo et al., 1995; Liu et al., 2000; Zhang and Ni, 1998]. With reference to our results obtained, the use of EA stimulation at ST36 could give more beneficial outcomes after transient focal cerebral ischemia. For examples, with the EA stimulation at ST36, the increased activities of superoxide dismutase and glutathione peroxidase were taking place as early as day 1 and maintained at day 4 after the transient focal cerebral ischemia (Figure 4.5 and 4.8). This led to the decreased levels of lipid peroxidation in terms of both MDA and 4-HNE (Figure 4.9). However, these effects were only restricted to day 1 after transient focal cerebral ischemia followed by EA stimulation at GB20. Moreover, greater increase in pBAD expression was only observed in the ischemic rats with EA stimulation at ST36 and none was detected in those with EA stimulation at GB20 (Figure 6.4). Therefore, our results may provide a further understanding about the effect of EA stimulation at two acupoints. In fact, the use of EA stimulation at ST36 is more common in treating various diseases, in which improvements were reported. For examples, patients with cerebral arteriosclerosis showed a reduced level of numbness after receiving EA stimulation at ST36 [Cheng, 1989]. The increased consciousness and muscle strength was observed in patients with cerebral hemorrhage followed by EA stimulation at ST36 [Li et al., 1989; Yang et al., 1999]. Experimental studies also found that the mRNA level of brain-derived neurotrophic factor (BDNF) was increased in the stress-induced animal with EA stimulation at ST36, which was important for normal development, survival and plasticity of neurons [Yun et al., 2002]. Therefore, in general our study suggests that the EA stimulation at ST36 could provide a better beneficial outcome on patients with stroke if applied clinically.
Table 9.2  Summary of the clinical outcomes after the use of EA stimulation on patients with stroke.

<table>
<thead>
<tr>
<th>Beneficial outcomes</th>
<th>EA stimulation at GB20</th>
<th>EA stimulation at ST36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase locomotion and muscle strength</td>
<td>Guo \textit{et al.}, 1995</td>
<td>Chen and Fang, 1990</td>
</tr>
<tr>
<td></td>
<td>Jiang \textit{et al.}, 1982</td>
<td>Pei \textit{et al.}, 2001</td>
</tr>
<tr>
<td></td>
<td>Tan, 1990</td>
<td>Ruan, 1980</td>
</tr>
<tr>
<td></td>
<td>Wang and Li, 2001</td>
<td>Si \textit{et al.}, 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xu and Cui, 1998</td>
</tr>
<tr>
<td>Improve speech</td>
<td>Cui, 1994</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jiang \textit{et al.}, 1982</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liu \textit{et al.}, 2000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zhao \textit{et al.}, 1998</td>
<td></td>
</tr>
<tr>
<td>Improve memory</td>
<td>Li and Shen, 1998</td>
<td></td>
</tr>
<tr>
<td>Improve paralysis</td>
<td>Jiang \textit{et al.}, 1982</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Qi \textit{et al.}, 1986</td>
<td></td>
</tr>
<tr>
<td>Improve cardiac blood flow</td>
<td>Jiang \textit{et al.}, 1982</td>
<td>Research School of</td>
</tr>
<tr>
<td></td>
<td>Wang \textit{et al.}, 1994</td>
<td>Chinese Medicine, 1979</td>
</tr>
<tr>
<td></td>
<td>Wang \textit{et al.}, 1996</td>
<td></td>
</tr>
<tr>
<td>Reduce cerebral infarction</td>
<td>Zou and Wang, 1990</td>
<td>Chen and Fang, 1990</td>
</tr>
<tr>
<td>Reduce permeability of blood vessels</td>
<td>Li and Shen, 1998</td>
<td></td>
</tr>
<tr>
<td>Reduce pain</td>
<td></td>
<td>Zhang \textit{et al.}, 2001</td>
</tr>
<tr>
<td>Reduce blood viscosity</td>
<td></td>
<td>Chen \textit{et al.}, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ji \textit{et al.}, 1987</td>
</tr>
</tbody>
</table>
9.4 LIMITATION OF THE STUDY

This study has investigated the effect of EA stimulation at GB20 or ST36 on regulating the reactive oxygen species-mediated toxicity and the apoptotic neuronal damage. However, there are still some limitations of the present study:

1. In relation to the experimental measurement:
   (i) The increased activity and expression has not been further proven by the administration of specific inhibitors.
   (ii) The localization of the proteins and co-localization of the expressed proteins were not obtained.
   (iii) The infarct volume of ischemic brains with or without EA stimulation was not measured.
   (iv) Similarly, the abundance of apoptotic bodies in brain tissues was not assessed.

2. In relation to the experimental design:
   (i) Significance of electrical stimulation was only investigated in the ischemic rats.

3. In relation to the treatment design:
   (ii) The treatment protocol (2Hz, 30 min, 0.7 V) has not been verified in terms of the frequency, duration of EA stimulation and the intensity of stimulation.
   (iii) The post-ischemia EA stimulation is only limited to a single application.
9.5 RECOMMENDATIONS FOR FURTHER STUDIES

In order to improve the present study, the following recommendations are suggested:

1. In relation to the experimental measurement:
   (i) The increased activity and expression is needed to further confirm by the intra-cerebral administration of specific inhibitors. Recommended inhibitors are wortmannin for the pAkt [Andjelkovic et al., 1997; Papapetropoulos et al., 2004], Z-Leu-Glu-ω-His-Asp-ω-FMK for the caspase-9 [Mouw et al., 2002], and L-NAME for the eNOS [Thomas et al., 2002].
   (ii) The localization of the proteins and co-localization of the expressed proteins can be identified by employing the immunohistochemical staining for specific proteins [Kitagawa et al., 1999].
   (iii) The infarct volume of the ischemic brains with or without EA stimulation was measured by staining with 2,3,5-triphenyltetrazolium chloride (TTC) in which the succinate dehydrogenase present inside the mitochondria of viable cells would turn the TTC substrate into red colour. If the infarct occurs, the brain may not be stained in red colour because of the inactivated succinate dehydrogenase [Benchoua et al., 2001].
   (iv) Similarly, the abundance of apoptotic bodies in brain tissues can be assessed by using the dUTP nick-end labelling (TUNEL) staining, which is specific for the *in situ* end-labelling of nuclear DNA fragmentation [Noshita et al., 2003].

2. In relation to the experimental design
   (i) The significance of electrical stimulation, two groups of normal rats with Sham EA stimulation and EA stimulation at each pair of bilateral acupoints
should be included in future study.

3. In relation to the treatment design:

   (i) The treatment protocol (2Hz, 30 min, 0.7 V) should be verified in terms of the frequency (such as a higher frequency 100Hz), duration of EA stimulation (15 min, 1 hour) and the intensity of stimulation (greater than or smaller than 0.7V which is the threshold intensity for muscle contraction).

   (ii) The effect of multiple post-ischemia EA stimulation should be investigated.
CHAPTER 10

Conclusion

The present study has investigated the effect of EA stimulation at Fengchi (GB20) and Zusanli (ST36) on regulating the reactive oxygen species-mediated injury and the apoptotic neuronal damage following the transient focal cerebral ischemia. The inhibition of activity and expression of nitric oxide synthase, together with the concomitant increase of superoxide dismutase and glutathione peroxidase, attenuates the lipid peroxidation. Protein oxidation is also prevented by potentiating the expression of thioredoxin and activity of thioredoxin reductase. Increased expressions of transforming growth factor β-1 (TGFβ-1), Bcl-2 and phosphorylated serine/threonine kinase (pAkt) serve to inhibit the dephosphorylation of BAD protein and the release of cytochrome c from the mitochondria, and subsequently suppress the activation of caspase-9 and caspase-3. Regarding the clinical consideration of the treatment, the comparable beneficial effects are also obtained after the delayed post-ischemia EA stimulation or multiple pre-ischemic EA stimulation. To conclude, the EA stimulation at GB20 or ST36 could ameliorate the ischemia-reperfusion injury, in which EA stimulation at ST36 produce a slightly better result.
Scanned copies of publication (1):


Electroacupuncture reduces the extent of lipid peroxidation by increasing superoxide dismutase and glutathione peroxidase activities in ischaemically-reperfused rat brains

Flora K.W. Siu, Samuel C.L. Lo, Mason C.P. Leung

Abstract

Reactive oxygen species can be scavenged by superoxide dismutase (SOD) and glutathione peroxidase (GPx). During ischemia-reperfusion, the normal function of these antioxidant enzymes may be insufficient for the prevention of calcium-induced peroxidation of micrometric lipids and hence cerebral infarction. We therefore investigated whether electroacupuncture (EA) treatment at Fengchi points in post-ischemic rat would increase the antioxidant enzyme activities and thereby reduce the extent of lipid peroxidation. The results indicated that while EA did not alter the antioxidant enzyme activities in non-ischemic control rat brains, ischemia-reperfusion caused significant increase in SOD and GPx activities. EA treatment further increased the antioxidant enzyme activities in ischemia-reperfused brain tissues, with a concomitant decrease in the extent of lipid peroxidation. Our finding suggests that EA treatment at Fengchi reduced the extent of lipid peroxidation in ischemia-reperfused rat brains, possibly by increasing the activities of SOD and GPx.

Keywords: Cerebral ischemia, Electroacupuncture, Glutathione peroxidase, Lipid peroxidation, Superoxide dismutase

Received 3 August 2003; revised manuscript received 6 October 2003; accepted 7 October 2003

Reactive oxygen species (ROS) have been implicated in neurotoxicity following cerebral ischemia-reperfusion. Accretion of ROS, particularly superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$), causes damage to membrane phospholipids, proteins and DNA [15]. Under normal circumstances, these oxidant species can be effectively scavenged by antioxidant enzymes, namely superoxide dismutase (SOD) and glutathione peroxidase (GPx). In mammalian cells, two forms of SOD are identified: copper-zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD) [16]. Both of them are able to convert $O_2^-$ to $H_2O_2$ [1]. The $H_2O_2$ formed is subsequently removed by the cytosolic GPxs to form water and molecular oxygen [18]. However, the sudden burst of ROS during cerebral ischemia-reperfusion can overwhelm the antioxidant defense, resulting in lipid peroxidation and hence cerebral infarction. In this regard, altered expressions of SOD or GPx might have significant implications in ischemia-reperfusion injury. Electroacupuncture (EA) has been shown to be able to reduce the extent of lipid peroxidation in ischemia-reperfusion injury, possibly by increasing the activities of SOD and GPx.

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ischemia-reperfusion injury, we investigated whether EA treatment could enhance cerebral SOD and GPX activities in animals subjected to experimentally-induced transient middle cerebral artery occlusion (MCAO). The extent of lipid peroxidation, an indirect index of tissue damage, was also examined. Male Sprague-Dawley rats weighing 350–350 g were housed in cages at animal facility (30 °C/12 h light cycle, 20–22 °C) and maintained with free access to food and water. Procedures were approved by the Animal Subject Review Sub-committee of The Hong Kong Polytechnic University. A total of 48 rats were divided into two experiments. Finally, 12 rats were used for the measurement of physiological parameters in blood obtained from the femoral artery 15 min before and during MCAO. Physiological parameters, including pH, partial pressure of oxygen (PaO₂) and carbon dioxide (PaCO₂), were determined by a Blood Gas Electrolyte Analyzer (Radiometer, ABL1, Copenhagen, Denmark). Secondly, 36 rats were assigned into four groups: Normal (n = 8), Normal-IA (normal rats with EA treatment, n = 4), Ischemia-IA (n = 8), Ischemia-Sham-IA (ischemic rats with Sham EA treatment, n = 8), and Ischemia-IA (ischemic rats with EA treatment, n = 8). Surgical procedures for the induction of MCAO were performed as previously described [16]. Rats were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg, Alfacain, Hollan) and xylazine (7 mg/kg, Alfacain, Hollan). Briefly, the temporalis muscle was separated in the plane of its fiber bundles to expose the zygomatic and squamous bone. A 5 × 5 mm burr hole was made using a saline-cooled electrical drill (Mortons, Dremel, Denver, USA). The exposed right MCA was occluded for 1 h followed by 24-h reperfusion. A single application of EA was conducted for 30 min following MCAO. For the Normal-IA, Ischemia-Sham-IA and Ischemia-IA groups, two stainless steel needles were bilaterally inserted into points corresponding to Fengshi points that were commonly used for treating stroke patients [19,20]. In human, Fengshi points lie on the posterior aspect of the neck, which are below the occipital bone and located in the depression between the sternocleidomastoid muscles and trapezius muscles [19]. In the rat, the Fengshi points were located 3 mm away from the center of a line joining the two ears. Electrical stimulation was delivered with EA stimulator (Model G-806-2, Medil, Shanghi, China) (voltage: 62.7 V, frequency: 2 Hz, duration: 0.5 ms) to animals in the Normal-IA, Ischemia-Sham-IA and ischemia-IA groups. No electrical stimulation was delivered in the Ischemia-Sham-IA group. Twenty-four hours following reperfusion, the right hemispheres of rat brains were removed, ovarian samples were weighed and homogenized in ice cold 20 mM Tris- HCl buffer (pH 7.4) with protease inhibitor cocktail (100X, Sigma, Saint Louis, MO, USA) in a volume of 70 μl per g (fresh weight) and the homogenates were centrifuged at 30,000 × g at 10,000 × g. The supernatants were collected for biochemical analysis. Total protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Total SOD activity was measured using xanthine-catalase as a substrate [11]. The superoxide-driven oxidation of xanthine-catalase was monitored spectrophotometrically at 555 nm. The enzyme activity was estimated by comparing the ratio of the oxidation rates in the presence (V1) and absence (V0) of SOD. One SOD activity unit was defined as the activity that decreased the oxidation rate of the control blank. SOD activity was assayed using an assay kit (Oxis International, Inc., Portland, OR, USA), as described [11]. Terr-butyl hydroperoxide was used as a specific substrate for GPX 112). The GPX-catalyzed reaction was measured for 5 min at 340 nm by spectrophotometer (Model U-2000, Hitachi, Japan). The GPX activity was expressed as nmol NADPH oxidized per mg protein. The assay did not measure the activity of glutathione-S-transferase (GST) which catalyzes the detoxification of organic nitrites and deactivation of nitrogensetodesin compounds [12]. Furthermore, brain tissues do not possess the highly active GST form which displays an independent GST peroxidase activity [4].

The extent of lipid peroxidation was determined by measuring the tissue level of the end products, namely, malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE), using an assay kit (Oxis International, Inc., Portland, OR, USA), which detected the formation of a chromophore with MDA or 4-HNE molecule, as described [5]. Both MDA and 4-HNE were measured because they were generated from different oxidative reactions [13,14]. The values were expressed as μM MDA or μM MDA/4-HNE per mg protein. Data were expressed as mean ± standard deviation. Statistical analysis of physiological parameters (PaO₂, PaCO₂, and pH) were performed using the patient t-test (SPSS version 11.2, Chicago, IL, USA). Comparisons among groups were made using analysis of variance (ANOVA) followed by post-hoc protected least-significant difference test. In any cases, it was considered to be statistically significant when P < 0.05.

As shown in Table I, arterial blood gas analysis of rats 15 min before and during transient focal cerebral ischemia indicated no differences in PaCO₂ and PaO₂. Likewise, the blood pH was not significantly altered before and during ischemia. These suggested that the experimental procedure adopted in the present study did not cause acidosis and that the observed changes in cerebral tissues were likely due to the ischemia-reperfusion challenge, as also reported in other studies [8].

As shown in Fig. 1, while EA treatment did not affect the

Table I

<table>
<thead>
<tr>
<th>Condition</th>
<th>PaO₂ (mmHg)</th>
<th>PaCO₂ (mmHg)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-ischemia (n = 8)</td>
<td>102.4 ± 9.4</td>
<td>42.2 ± 3.4</td>
<td>7.34 ± 0.07</td>
</tr>
<tr>
<td>Post-ischemia (n = 8)</td>
<td>103.2 ± 5.7</td>
<td>42.8 ± 3.3</td>
<td>7.34 ± 0.08</td>
</tr>
</tbody>
</table>

Data are mean ± S.D. PaO₂, partial pressure of oxygen; PaCO₂, partial pressure of carbon dioxide.
cardiac total SOD activity in non-ischemic normal rats, ischemia-reperfusion caused a significant increase (31%) in total SOD activity. EA treatment further increased the total SOD activity in ischemic-reperfused rat brains, with the value being significantly higher than those of the ischemic-reperfused and ischemic-reperfused Sham EA groups.

Fig. 2 shows the GPx activity in non-ischemic and ischemia-reperfused rat brains, with or without EA treatment. While EA treatment did not affect the GPx activity in non-ischemic brains, ischemia-reperfusion increased the GPs activity by 5.5. As similar to SOD activity, EA treatment produced a further increase in GPr activity, with the value being significantly higher than those of the ischemic and ischemic-reperfused Sham EA groups.

As shown in Fig. 3, the cerebellum MDA level, but not the total MDA-HNE level, was increased by 15-20% in EA-treated and non-ischemic rat brains. Ischemia-reperfusion caused significant increases in both MDA (105%) and total MDA-HNE (58%) levels. EA treatment significantly decreased both MDA and MDA-HNE levels in ischemia-reperfused rat brains, when compared with those of the ischemic, control and ischemic-reperfused Sham EA groups.

Results obtained from the present study agree well with other studies, showing that both MnSOD and CuZnSOD activities could be increased by transient focal cerebral ischemia. It was found that the upregulation of enzyme activities started as early as 4 h and reached the maximum at day 1 after ischemia. In this regard, the increased SOD activity may play a protective role in development of ischemic tolerance and the survival of neurons after ischemia [9]. Similarly, the ischemia-reperfusion associated increase in GPr activity, as observed in our study, was also in line with the finding reported by other workers [11]. The increase in GPr activity may reveal a compensatory response to the increase in H$_2$O$_2$ production arising from the SOD-catalyzed reaction [10]. However, the enhanced SOD and GPr activities were not associated with the suppression of lipid peroxidation in ischemia-reperfused brain tissues. Consistent with this, Kondo et al. have reported that both MDA and 4-HNE levels were increased in frontal cortex and hippocampus within 1 day after transient focal cerebral ischemia [11]. The extent of ischemia induced increase in activities of SOD and GPr, as observed in the present study, may not be sufficient for inhibiting lipid peroxidation.

Increasing the activities of antioxidant enzymes such as SOD and GPr can undoubtedly ameliorate the extent of lipid peroxidation. Our results showed that EA treatment applied after cerebral ischemia could increase both SOD and GPr activities, resulting in a lesser extent of lipid peroxidation. In other traumatic injuries or neuronal diseases where EA was used as a form of treatment, an upregulated SOD activity with decrease in MDA contents was observed [12, 13]. However, the involvement of GPs in the protective mechanism of EA has not been reported. Low expression and activity of catalase in brain tissues diminish its role in removing H$_2$O$_2$ [13]. In contrast, GPs was found to have a higher tolerance to ischemic or hypoxic insults [14, 16]. Thus the removal of H$_2$O$_2$ or phospholipid hydroperoxides in brain tissues was mainly dependent on the GPs activity. In the presence of high SOD activity, excessive H$_2$O$_2$ is formed from O$_2$, and this would result to a larger extent of lipid peroxidation if the GPs activity is not increased in parallel [1, 17]. In this regard, EA treatment likely provides an appropriate antioxidant balance by increasing both SOD and GPs activities, thereby reducing the extent of lipid peroxidation in ischemia-reperfused brain tissues.

The application of electrical stimulation was found to produce neuro-protective effect. A single application of EA, not manual acupressure, on P6 acupoints was able to
regulate the cerebral blood flow and pressure (19). In the present study, neither antilipidemic enzyme activators nor lipid peroxidation was modified in animals subjected to cerebral ischemia with sham RA treatment, in which no electrical stimulation was given to the insert electrode. This suggests that RA treatment may produce additional beneficial effects than manual acupressure.

In conclusion, our results demonstrated that RA could reduce the extent of lipid peroxidation in ischemic-reperfused brain tissues, possibly by increasing both SOD and GSH activities after transient focal cerebral ischemia in rats.

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Reference


Scanned copies of publication (2):


Effectiveness of multiple pre-ischemia electro-acupuncture on attenuating lipid peroxidation induced by cerebral ischemia in adult rats

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Abstract

Free radicals induced by cerebral ischemia-reperfusion injury can trigger lipid peroxidation, leading to the production of malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE). Post-ischemia electroacupuncture (EA) therapy was able to reduce extent of lipid peroxidation. However, the effect of pre-ischemic EA therapy has not been reported. In this study, we aim to investigate the effectiveness of pre-ischemic EA therapy on lipid peroxidation in the rat ischemic injury model. Four groups of Sprague-Dawley rats were designed. Placebo group (without EA therapy), NA group (EA therapy on non-acupoint), GB20 group (EA therapy on P6acupoint) and ST36 group (EA therapy on Zusanli). Half of each group (n = 6) received 30-minute EA therapy for 3 times and the other half group for 18 times before the occlusion of right middle cerebral artery. Right brains were taken for determination of concentration of MDA and the total of MDA plus 4-HNE. We found that multiple pre-ischemia EA therapy at either GB20 or ST36 can effectively reduce the amount of MDA produced after MCA occlusion. However, this reduction was not observed in the total amount of MDA and 4-HNE. In conclusion, pre-ischemia EA can partly regulate the lipid peroxidation in cerebral ischemia, where both GB20 and ST36 have a similar beneficial effectiveness.

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Keywords: Electro-acupuncture; Cerebral ischemia; Lipid peroxidation

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Introduction

Lipid peroxidation is triggered by high levels of free radicals which can be induced in the cerebral ischemia-reperfusion model (Cheeseman and Slater, 1993; Esteban et al., 1987, 1991; Konno et al., 1988). Determination of its products, malondialdehyde (MDA) and 4-hydroxy-2(5H)-enone (4-HNE), in brain tissues and plasma has been used to evaluate the severity of neuronal injury of ischemia (Bremner et al., 1989; Kondo et al., 1997; Re et al., 1997). Both MDA and 4-HNE are cytotoxic to cells but their reactivities to biomolecules are different (Cheeseman and Slater, 1993). MDA demonstrates its specific reactivity on proteins, while 4-HNE has its specificity on DNA and membrane, thus inducing apoptosis (Choudhury et al., 2002). For example, at a concentration of 4 mM, MDA nearly completely inhibited the total enzyme activity of xanthine oxidoreductase, whereas HNE could partially inhibit it (Gigliotti et al., 2001). On the other hand, only 4-HNE was effectively able to decrease membrane fluidity and inhibit the mitochondrial transmembrane at millimolar concentration (Chen and Yu, 1994; Krestal et al., 1994).

Acupuncture, which is an insertion of acupuncture needles into correct acupoints, has been used for treating various diseases in oriental countries (Leake and Broderick, 1998). Acupoints are located along the skin with the properties of lower electrical impedance, higher electrical potential, and more peripheral nerves than other areas of the skin (Rosenthal, 1981; Leake and Broderick, 1998). The ultimate goal of acupuncture, by different acupuncture techniques is to restore the internal balance and harmony (Leake and Broderick, 1998). One form of techniques is manual acupuncture which is the manual manipulation of acupuncture needles at acupoints. Another form is electroacupuncture (EA) that delivers electrical stimulation to the acupoints through the acupuncture needles. In particular electroacupuncture (EA) has been more recommended as the complementary therapy for pain relief (Konno et al., 1997; Tsubi and Leang, 2002) and stroke rehabilitation in both Asian and western countries (Cheng, 1987; Wu and Xiao, 1998). A considerable number of studies had been conducted on investigating the effectiveness of EA therapy on patients with cerebral ischemia. Several beneficial outcomes were observed, such as reduced paralysis by increasing muscle strength (Huang et al., 1998; Xing and Zhang, 1998), improved speech ability (Xing and Zhang, 1998), reduced mental retardation (Xing and Zhang, 1998), restored cerebral blood flow (Tan, 1990; Di et al., 1993) and improved locomotion (Tan, 1990; Chang et al., 1996; Xing and Zhang, 1998; Liu et al., 1998). These observations support the hypothesis that post-ischemic EA can be an effective modality of treatment in stroke condition. However, little is known about the effectiveness of pre-ischemic EA on minimizing the neurological injury caused by stroke.

Our experimental study aims to investigate the effect of multiple applications of EA therapy before the induction of cerebral ischemia. There are two acupoints selected for this study, Fengchi (GB20) and Zusani (ST36). Fengchi, which has been an effective acupoint selected for stroke rehabilitation, has produced a greatly enhanced locomotion ability of stroke patients (Tan, 1990; Liu et al., 1999). On the other hand, Zusani is regarded as a common acupoint treating various diseases (Yin et al., 2002). Experimental studies showed that EA therapy on ST36 was able to stimulate cell proliferation in dopamine neurons after transient global ischemia in gerbils (Kim et al., 2001). It is also capable of stimulating the expression of brain-derived neurotrophic factor (BDNF) in rat hippocampus leading to the relief of neuropathological stress (Yin et al., 2002). The increase of BDNF concentration in hippocampus is also associated with the recovery of neuronal function (Chen and Fang, 1990). In addition, stroke patients with EA therapy on both GB20 and ST36 restored their speech ability and...
reduced the amount of plasma lipid peroxides formed (Li and Shua, 1998; Zhao et al., 1998). In order to address the significance of selection of acupoints, bilateral non-acupoints were additionally selected for comparison. After the induction of cerebral ischemia, outcomes of EA therapies on different acupoints were assessed by quantifying the amount of both products of lipid peroxidation, MDA and 4-HNE.

Materials and methods

Experimental conditions

A total of 64 male Sprague–Dawley rats 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. Secondly, four rats were assigned as the Normal group and forty-eight rats were evenly assigned to four groups: (1) Control group (rats with anesthesia but without EA), (2) Non-acupoint (NA) group (rats with EA stimulation on non-acupoint), (3) GB20 group (rats with EA stimulation on Fengchi, GB20), and (4) ST36 group (rats with EA stimulation on Zusanli, ST36). EA stimulation was carried out on alternative days before the induction of cerebral ischemia. In the Control group, however, only general anesthesia was used. In each group, half of the rats (n = 6) were subjected to 3 times of EA conducted for a week and another six rats were subjected to 18 times of EA conducted in 6 consecutive weeks. All the rats were then induced with transient focal cerebral ischemia. Rat brains were harvested at post-ischemia day 4.

Pre-ischemia electro-acupuncture

A thirty-minute EA was applied by an acupuncture-needle device (Model G6805-2, Shenf, Shanghai, China). In the GB20 and ST36 groups, EA was applied on bilateral points corresponding to GB20 or ST36, respectively. GB20 is anatomically located on the posterior aspect of the neck, below the occipital bone, in the depression between the sternocleidomastoid muscle and trapezius muscle (Ellis et al., 1991). ST36 is anatomically located near the knee joint of the hind limb 2 mm lateral to the anterior incisura of the tibia (Ellis et al., 1991). In the NA group, EA was applied on non-acupoint which was located midway between coccyx and hip joints. Fig. 1 illustrates the location of acupoints used. A frequency of 2 Hz with a square waveform at pulse duration of 0.5 ms was used. Voltage applied was 0.7 mV that was the intensity just below the threshold of muscle contraction.

Production of transient focal cerebral ischemia

All experimental procedures described below were approved by the Animal Ethics Sub-committee of The Hong Kong Polytechnic University. Transient focal cerebral ischemia was induced by occlusion of right middle cerebral artery (MCA) for 1 hour as described by Chen et al. (1986) with modifications (Leung et al., 2002). Rats were anesthetized with intraperitoneal injection of ketamine (10 mg/kg, Alfasan, Holland) and xylazine (7 mg/kg, Alfasan, Holland). Briefly the temporal muscle was separated in the plane of its fiber bundles to expose the zygomatic and squamosal bone. A 3 mm × 5 mm hair hole was made using a saline-cooled electric drill (Motorex, Dremel, Denver, Colo).
Exposed MCA was occluded for 1 hour followed by reperfusion. During the surgical procedures, rectal temperature of rats was monitored and maintained at about 37°C with an overhead lamp. After recovery from experimental surgery, all rats were allowed to recover at ambient temperature (21–23°C) with food and water made available ad libitum until harvest. For the measurement of physiological parameters, arterial blood samples were taken from the femoral artery 15 min before and after MCA occlusion for the determination of pH, arterial partial pressure of oxygen (PaO₂) and carbon dioxide (PaCO₂) by a Blood Gas and Electrolyte System (Radiometer ABL505, Copenhagen, Denmark).

Lipid peroxidation assay

Unless stated otherwise, all materials were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO, USA). At post-stenosis day 4, rats were sacrificed after anesthesia with ketamine and xylazine. Transcardiac perfusion was performed with 0.38% sodium citrate in normal saline for 10 min followed by normal saline for 5 min. The harvested right hemisphere of the brain was weighed and homogenized mechanically with an Ultra-Turrax T25 homogenizer in 9 volumes of 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. Protein concentration was determined by using protein assay as described by Bradford (1976) (Bio-Rad Laboratories, Hercules, CA, USA). Malondialdehyde (MDA) and 4-hydroxy-2(1H)-nitroso(4-HNE) were determined by using a colorimetric assay as described by Endo et al. and co-workers (1998) (OxS International, Inc., Portland, OR, USA). In brief, each sample mixture was composed of 50 μl of supernatant, 59 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 and 4-HNE, sample mixture was added with 75 μl of 15.4 M methanesulfonic acid and incubated at 45°C for 40 min. To assay for the quantity of MDA only, sample mixture was added with 75 μl of 12 N HCl and incubated at 45°C for 1 hour. After incubation, samples were cooled on ice and their absorbance was read at 586 nm using a microtiter plate reader (Winooski, Vermont, USA). The unit
was defined as [MDA] (μM) per mg homogenate protein or [MDA + 4-HNE] (μM) per mg homogenate protein.

Statistical analysis

The physiological parameters obtained from blood and amounts of LPX products were expressed as means ± one standard deviation (SD). The arterial blood values for pCO₂, pO₂, and pH were analyzed by paired t-tests using SPSS Version 11.0 (SPSS, Chicago, IL, USA). To analyzing the quantity of products of lipid peroxidation, ANOVA followed by post-hoc test, LSD, was performed to analyze different groups at the same EA treatment times or different treatment times at the same group. In all instances, P < 0.05 was considered statistically significant.

Results

Physiological parameters

Physiological data obtained 15 minutes before and during transient focal cerebral ischemia are shown in Table 1. These parameters were comparable to previous studies (Lotar et al., 2001), implying that the ischemia-reperfusion model was successfully established. Difference between pO₂ measured before ischemia (103.3 ± 7.77 mmHg) and after ischemia (103.3 ± 5.69 mmHg) was not statistically significant (t = 0.776, degrees of freedom (d.f.) = 5, α = 0.05). The pCO₂ levels measured before and after ischemia (43.5 ± 4.84 mmHg and 43.2 ± 4.74 mmHg, respectively) showed no significant difference (t = 0.824, degrees of freedom (d.f.) = 5, α = 0.05). In addition, there was no observable difference in blood pH. Changes in physiological status were not revealed suggesting that change of lipid peroxidation was not a result of physiological changes.

Lipid peroxidation

Changes in total concentration of MDA and 4-HNE in the rat right brain after multiple pre-ischemia EA therapy were shown in Fig. 2. The basal value of MDA and 4-HNE obtained from the Normal group was 13.38 ± 1.05 μM per mg protein. After either pre-ischemia EA therapy for 3 or 18 times, only the NA group produced greater concentrations of MDA and 4-HNE than the Normal group did. In the 2-times EA therapy before cerebral ischemia, the NA groups showed a remarkably

Table 1

<table>
<thead>
<tr>
<th>Physiological data from rats subjected to transient focal cerebral ischemia</th>
<th>pO₂ (mmHg)</th>
<th>pCO₂ (mmHg)</th>
<th>pH (unit)</th>
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</thead>
<tbody>
<tr>
<td>Pre-ischemia (n = 6)</td>
<td>103.1 ± 7.77</td>
<td>43.3 ± 4.84</td>
<td>7.22 ± 0.0012</td>
</tr>
<tr>
<td>Post-ischemia (n = 5)</td>
<td>103.1 ± 5.69</td>
<td>43.2 ± 4.74</td>
<td>7.23 ± 0.0017</td>
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</table>

Data (mean ± standard deviations) obtained from arterial blood at 15 min before and after ischemia. Abbreviations: pO₂, partial oxygen pressure; pCO₂, partial carbon dioxide pressure.
increased amount of MDA and 4-HNE when comparing to the GB20 and ST36 groups (p < 0.01). The mean value of GB20 and ST36 groups was only 55% of that of the NA group. However, this increase was not observed when the 18-times EA therapy was used. In addition, the amount of MDA and 4-HNE of the each group with different number of EA therapy was not found to be statistically significant (p > 0.05). It suggested that the number of EA therapy was not critical to attenuate the lipid peroxidation.

Changes in MDA concentration in the rat right brain after multiple pre-ischemia EA therapy were shown in Fig. 3. Only the Control group in the 3-times EA therapy and NA groups at both frequencies of EA therapy produced more than two-times of MDA concentration (p < 0.05) than the Normal group (4.87 ± 0.44 μM per mg protein). On the other hand, significant difference was observed when comparing concentrations of MDA between groups of each treatment frequency. In the 3-times EA therapy before cerebral ischemia, the NA groups showed a remarkably increased MDA amount in comparison to the Control group (p < 0.05). Further, its concentration is four-times that of the GB20 group and three-times that of the ST36 group (p < 0.05). Only the GB20 group but not the ST36 group, produced nearly 40% of the concentration of MDA of the Control group (p < 0.05). Similarly, the NA group in the 18-times EA therapy had the highest MDA amount detected, which was two-fold greater than those of GB20 and ST36 groups. It may imply that the use of non-acupoint would exacerbate the lipid peroxidation. Although the GB20 and the ST36 groups produced lesser MDA amount than that of the Control group (p < 0.05), there was no significant difference
between them. These may suggest that both the GR20 and ST36 groups could effectively regulate the MDA production. Comparison of MDA amounts of the same groups with two different frequencies of EA therapy found that there was no significant difference. This suggested that the increased frequency of EA therapy before cerebral ischemia was not related to the decreased MDA production.

Discussion

Both MDA and 4-HNE are cytotoxic to neuronal cells. 4-HNE exhibited a wider specific reactivity on DNA and membrane, while MDA only had specificity on proteins. However, the production of MDA was more rapidly and dominantly induced in different brain regions and plasma under the ischemia-reperfusion model. For example, in the four-vessel occlusion model, MDA level was not increased until reperfusion started (Bruno et al., 1999). During the first hour of reperfusion, MDA levels were increased in the cortex, hippocampus, and striatum. Before 72 hours of reperfusion, a substantially increasing amount of MDA was observed only in cortex. After 72 hours, the MDA level in all regions increased with the time of reperfusion. Similarly, plasma samples from stroke patients were detected with an increased level of MDA but not the total level of MDA and 4-HNE (Re et al., 1997). Up-regulated MDA level was likely as a result of increased activities of neutrophils in inflammation caused
by cerebral ischemia (Ke et al., 1997). Therefore any therapy capable of reducing MDA amount was served to be more beneficial to neuronal survival.

This study has compared the effect of EA therapy at GB20 and ST36 on ameliorating the lipid peroxidation levels. Our results showed that EA therapy at either acupoint could reduce the level of MDA production and restore it to the normal level. Either at the 3-times or 18-times EA therapy, the lowest amounts of MDA were observed in the GB20 groups, which were half of values in the Control group (p < 0.05). This significant reduction was only observed in the ST36 group after 18-times pre-ischemic EA therapy. Our results are in line with the reduced cerebral infarction, which was as a result of lipid peroxidation, after EA therapy at either GB20 or ST36 (Chen and Fang, 1990; Zou and Wang, 1990). In the case of the functional abilities, such as locomotion (Liu et al., 1999) and speech ability (Zhang and Ni, 1998), stroke patients with EA therapy at GB20 on stroke patients had a greater extent of recovery than those with EA therapy at ST36. However, our statistical analysis revealed that there was no observable difference of MDA amount between the GB20 and ST36 group. It may imply that both EA therapy at GB20 or ST36 could produce a comparable and beneficial affect at the cellular level.

In addition, the significance of the selection of appropriate acupoints was demonstrated by the NA groups (rats with EA therapy at non-acupoints). The non-acupoints were bilaterally located near to a pair of well-known acupoint, Huanliao (GB30). Huanliao is located on the outer 1/3 distance between ep of coccyx and hip joints (Ellis et al., 1991). Previous studies found that the amount of MDA in the ischemic brain of adult rat was decreased after EA therapy on Huanliao (Chen et al., 1998). However, total amount of MDA and 4-HNE produced as well as the amount of MDA alone were found to be the highest in the NA groups. It was about 30% more than the values found in the Control group (p < 0.05). These results implied that the non-acupoint used was highly unlikely to be Huanliao. It further suggested that wrongly locating the acupoints and providing EA therapy on non-acupoints may be detrimental to the health of stroke patients.

Nevertheless, when measuring both MDA and 4-HNE, the EA therapy at either GB20 or ST36 could reduce their amounts as comparable as the Control group. This failure was unlikely associated with the reliability of the assay method. The used method showed much less interference than that by the determination of thiobarbituric acid reaction substance (TBARS) previously used (Eichlin et al., 1988; Jünger, 1998). The failure may be partly related to the time of measurement. Anthom et al. (1997) found that level of MDA and 4-HNE could only be enhanced in the frontal cortex and hippocampus within 1 day after transient focal cerebral ischemia. Except the striatum, none of the brain regions were found to increase the total amount of MDA and 4-HNE during 1 week to 24 weeks after ischemia. It may suggest that harvest at post-ischemia day 4 was not an appropriate time for the measurement of total MDA and 4-HNE. Moreover, the number of animals used may also contribute to the failure. Statistical analysis revealed that the observed power of this measurement was only 0.481, whereas the observed power of measurement of MDA only was 0.961. These findings suggested that more animals should be recruited for the measurement of total lipid peroxidation products.

Unlike previous studies that focused on the stroke rehabilitation by EA stimulation, our study can provide another view of clinical application of EA therapy. The pre-ischemic EA therapy is particularly important to those high-risk groups such as patients with high blood pressure. Keeping EA stimulation at either GB20 or ST36 at alternative days per week may ameliorate the severity of brain damage after stroke attack, so as to shorten the time of the recovery.
Conclusion

To conclude, multiple pre-injury EA therapy at either GB20 or ST36 can effectively reduce the amount of MDA resulted from lipid peroxidation induced by cerebral ischemia-reperfusion. According to the specific reactivity of MDA, reducing MDA amount by EA therapy may promote the production of protein against oxidative stress. Owing to the limited sample size, this beneficial effect was not truly revealed in measuring the total amount of MDA and 4-HNE. Further experiment recruiting a large animal size for the measurement of total MDA and 4-HNE is suggested.

Acknowledgements

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References


Electro-acupuncture potentiates the disulphide-reducing activities of thioredoxin system by increasing thioredoxin expression in ischemia-reperfused rat brains

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Abstract

Reactive oxygen species can directly affect the conformation and activity of sulphydryl-containing proteins by oxidation of their thiol moiety. During the process of ischemia-reperfusion, the thioredoxin (Trx) system (consisting of thioredoxin reductase (TR), Trx and NADPH) prevents susceptible proteins from this oxidative modification. Oxidative damage is one of the most damaging stress in ischemia. If oxidative stress could be minimized, the damage occurred will be minimized accordingly. We therefore investigated whether electroacupuncture (EA) treatment at Fengchi (GB20) or Zuanti (ST36) acupoints in post-ischemic rats could increase Trx-related activities and Trx expression which would translate into maintaining the intact thiol moiety of susceptible proteins in the surrounding. Our results indicated that EA treatment at either acupoint increased the Trx expression in ischemia-reperfused brain tissues. Indeed, Trx expression levels gradually increased from post-ischemia day 1 to day 4. Statistical analysis revealed that there was no observable difference in the effect of EA treatment at GB20 and ST36. Sham EA treatment did not induce any Trx expression. EA at either acupoint did not alter TR activities in both non-ischemic and ischemia-reperfused rat brains. Taken overall, our finding suggests that EA treatment at GB20 or ST36 could increase Trx expression which could minimize oxidative modifications of thiol groups of surrounding protein.

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Keywords: Cerebral ischemia; Electroacupuncture; Thioredoxin; Thioredoxin reductase

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Introduction

Protein thiol reactivity has always demonstrated essential structural, regulatory, and catalytic functions in the cellular system. Under normal circumstances, a stable intracellular thiol-redox environment is maintained as to keep thiols groups from oxidation (Nordberg and Amor, 2001). However, a huge production of reactive oxygen species (ROS) during ischemia-reperfusion alters a properly balanced thiol-redox environment, resulting in the oxidation of protein thiols of some enzymes, resulting in the loss of their normal biological activities (Butterfield et al., 1994; Stadtman and Berlett, 1998). The undesired oxidation of protein thiols can be reversed by the thioredoxin system (Nordberg and Amor, 2001). The thioredoxin system consists of thioredoxin (Trx), thioredoxin reductase (Trx) and NADPH (Luthra and Holmgren, 1982). It promotes the reduction of ROS-induced protein disulfides by NADPH through a series of reactions as described in Equations (1) and (2).

\[
\text{Thioredoxin-S}_{\text{2}} + \text{NADPH} + \text{H}^{+} \rightarrow \text{Thioredoxin-(SH)}_{\text{2}} + \text{NADP}^{+}
\]

\[
\text{Thioredoxin-(SH)}_{\text{2}} - \text{Protein-S}_{\text{2}} \rightarrow \text{Thioredoxin-S}_{\text{2}} + \text{Protein-(SH)}_{\text{2}}
\]

The thioredoxin system displays its antioxidant activities by the TR-directed and Trx-mediated reduction of the disulfide groups by TR. In ischemia-reperfusion studies, it was found that thiolation TR can directly reduce the S-nitrosothiol (SNO) formed after the reaction of glutathione (GSH) with nitric oxide (Burton, 1985; Gaston et al., 1993). Trx was used by TR in this reaction and hence it is usually regarded as antioxidant. Reduced Trx has been shown to efficiently scavenge hydrogen peroxide and hydroxyl radicals (Nakamura et al., 1994; Takagi et al., 1996; Gerner et al., 1999; Ando and Holmgren, 2000; Mustafah and Powis, 2000; Powis, 2002). Also, thioredoxin superoxide dismutase (Hafner et al., 1994; Björnstedt et al., 1995). Moreover, it can reactivate the thiols groups on glyceraldehyde-3-phosphate dehydrogenase which had been inactivated by ROS (Fernando et al., 1992; Nakamura et al., 1994). In addition, reduced Trx can inhibit the apoptotic process by inducing the expression of Bcl-2, suppressing the release of cytochrome c and the expression of caspase-3 and-9 (Ando et al., 2002), and blocking the MAP kinase/p38 pathway via an inactivated apoptosis signal-regulating kinase (ASK-1) (Sadah et al., 1998).

Acupuncture, a technique of stimulation on acupoints by acupuncture needles, has been used for many years in Asian countries. Acupoints are located along the skin and featured with lower electric impedance, higher electrical potential, and more peripheral nerves than other areas of skin (Koebnick, 1984; Leake and Bredéricht, 1994). One associated technique is manual acupuncture (MA) in that mechanical stimulation is given to acupoints. In another technique called electroacupuncture (EA), electrical stimulation is delivered to acupoints. Rather than MA, EA has been shown to produce clinically beneficial effects on stroke patients, such as its ability to minimize speech retardation (Zhang and Ni, 1998; Zhao et al., 1998; Liu et al., 2000), improve locomotion and increase muscle strength (Tan, 1995; Zhang and Ni, 1998; Liu et al., 1999), and enhance memory (Li and Shen, 1998) and stimulate cerebral blood flow (Research School of Chinese Medicine, 1997; Jiang et al., 1992; Wang et al., 1994; Wang et al., 1996). On the other hand, experimental studies reported that EA could effectively attenuate edema formation, lipid peroxidation and cerebral infarction (Xu et al., 1996; Chu et al., 1999; Zhao et al., 2000). The stimulating effect likely resulted from the reduced production of nitric oxide (Xu et al., 1996; Zhao et al., 2000), and increased activities of superoxide dismutase and glutathione peroxidase...
(Su et al., 2004), and hence its suppression of lipid peroxidation. Nevertheless, it remains unclear if EA could regulate the activities of TR and the expression of Trx because both were usually highly abundant in nerve tissue (Rozell et al., 1995; Sommer et al., 1985; Hoppold, 1995).

The present study aims to investigate if EA could increase TR activity or potentially Trx expression in the ischemic rat brains. Two approaches were selected for this study, Fengchi (GB20) and ZuSanLi (ST36). Fengchi has been recognized as an effective acupoint for stroke rehabilitation (Tan, 1990; Liu et al., 1990). ZuSanLi is regarded as a common acupoint for treating neurological diseases (Chen and Fang, 1990; Lam et al., 2001; Su et al., 2002). In order to address the significance of electrical stimulation, two groups of rats with Sham EA (Sham-EA) were used as control. After the induction of cerebral ischemia, the activities of TR on reducing protein disulfides and the expression of Trx were determined.

Methods

Experimental design

Male Sprague-Dawley rats weighing 330-350 g were maintained with free access to food and water under a controlled environment (12 h dark/12 h light cycle, 20-22°C). All experimental procedures were approved by the Animal Subject Ethics Sub-committee of the administering institution. A total of sixty-six rats were randomly assigned into eleven groups. The induction of cerebral ischemia and EA stimulation given to each group (n = 6) were summarized in Table 1. Since previous studies reported that EA stimulation on normal rats would not stimulate the increased production of brain enzyme and mRNA expressions, the group of normal rats with EA or sham EA stimulation was not included (Wei et al., 2000; Kao et al., 2001; Su et al., 2004).

Induction of focal cerebral ischemia

Transient focal cerebral ischemia was induced by the occlusion of right middle cerebral artery (MCA) for 1 hour as previously practiced (Su et al., 2004). Rats were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg, Alfasan, Holland) and xylazine (7 mg/kg, Alfasan, Holland). The temporalis muscle was separated in the plane of its fiber bundles to expose the zygoma and squamous bones. A 3 mm x 5 mm burr hole was made using a saline-cooled electric drill (Metomed, Dremsel, Duren, Cello). The exposed MCA was occluded for 1 hour followed by reperfusion. During the surgical procedures, the rectal temperature of rats was monitored and maintained at about 37°C with an overhead lamp. After recovery from experimental surgery, all rats were allowed to recover at ambient temperature (21-23°C) with food and water made available ad libium until harvest.

Electroacupuncture stimulation

A single EA stimulation was applied for 30 min following MCAO. Two acupuncture points commonly used for treating stroke patients were selected. For the rats receiving Sham EA-GB20 or EA-GB20, two stainless steel needles were bilaterally inserted into points corresponding to Fengchi.
Table 1: Design of grouping of rats for the induction of transient cerebral ischemia and EA stimulation.

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<th>Post-ischemia Day 1</th>
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Transient cerebral ischemia was induced by right middle cerebral artery occlusion (MCAO) for 1 hour followed by reperfusion. Rats designated for Sham EA-GB20 and Sham EA-ST36 treatments were injected with acupuncture needles at P6, Neiguan (GB34), and Zusanli, (ST36), respectively, but without electrical stimulation. Rats assigned for EA-GB20 and EA-ST36 received electrical stimulation at bilateral acupuncture points, P6 and Neiguan, respectively. After MCAO, a single electrical stimulation was applied at voltage 0.7 N, frequency 2 Hz, duration 0.5 ms for 30 min. To identify the brain protein expression and activity, a group of normal, healthy rats was included and assigned to the Normal group (N). Right brains of rats were observed at either postischemia day 1 or day 4.
points (GB20). In humans, P69uchi is located on the posterior aspect of the neck, below the occipital bone, in the depression between the sternocleidomastoid muscles and trapezius muscles (Gills et al., 1991). In rats, the points were located 3 mm away from the centre of a line joining the ears. For the new receiving Sham EA-ST36 or EA-ST45, two needles were bilaterally inserted into points representing Zusanli points (St35). In humans, Zusanli is located 3 cm below the knee joint of the hind limb and 1 cm lateral to the crest of the ilia (Gills et al., 1991). In rats, the points were located 5 mm below the knee joint of the hind limb and 2 mm lateral to the anterior tubercle of the tibia. Electrical stimulation was delivered with an EA stimulator (Model G808-O2, Sencor, Shanghai, China) at voltage 0.7 V, frequency 2 Hz, duration 0.5 ms, to the groups receiving it at EA-CB30 (1-G and 11-G) and EA-ST36 (11-ST and 14-ST). No electrical stimulation was delivered to ischemic rats with Sham EA stimulation (11-SG3, 14-SG3, 11-ST and 14-ST).

Preparation of brain homogenate

Unless stated otherwise, all materials were of analytical grade and obtained from Sigma Chemical Co. (Saint Louis, MO, USA). After 1 day and 4 days of superfusion, the right hemisphere of brains of each group was removed, weighed, homogenized in 2 volumes of ice-cold 20 mM Tris-HCl buffer (pH 7.4) with protease inhibitor cocktail (1:140, Sigma Co.) and centrifuged for 30 min at 10,000 g. The supernatant was collected for biochemical analysis. Total protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

TR activity on DNP reduction

Mammalian TR has broad substrate specificity. In the presence of NADPH, it can directly reduce small, disulfide-containing molecules, such as 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ludhian and Holmgren, 1982). The reaction produced the yellow-coloured monomers, 2-nitro-5-thiobenzoic acid (TNB), with a maximum absorbance at 412 nm. Brain homogenate (100 μl) was added with 0.9 ml assay mixture (100 mM K2HPO4, 3H2O, pH 7.13, 10 mM EDTA, 5 mM DTNB, 0.2 mM NADPH) into a cuvette. The reaction was subsequently monitored at 412 nm for 2 min by a spectrophotometer (Model U-2000, Hitachi, Japan). As 1 mole of NADPH yielded 2 moles of TNB, a correction factor of 0.0184 derived from 0.5 × (13.5 × 2) was made. Hence we calculated TR activity using 3A412/min × 0.0184. The activity unit was defined as moles NADPH oxidized per minute per mg protein.

TR activity on the reduction of immunoglobulin molecules

In addition, with the aid of Txx and NADPH, TR can reduce the protein disulfide bridges of larger molecules, such as immunoglobulin G (IgG) employed in this study. This method relies on its reducing action on the inter-heavy-light chain and inter-heavy chain disulfides of IgG (Magunov et al., 1977). The functional activity level of brain TR was measured using commercially available Txx obtained from Spirulina species (T3658, Sigma Co.). A positive control was included using the purified TR obtained from rat liver (Activity level was 14 pmol NADPH oxidized per min per mg protein). In 50 μl incubation mixture, 10 μl brain homogenate was incubated with 200 μg thiobenzoic acid IgG, 5 μM Spirulina Txx and 50 μM NADPH in 20 mM Tris-HCl (pH 7.4) for 30 min at
37°C with gentle shaking. The incubated reaction mixture was boiled with 50 μL 1X working non-reducing SDS-PAGE sample buffer (62.5 μM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% bromophenol blue). The boiled reaction mixture was then resolved by SDS-PAGE using 10% separating gel and 4% stacking gel in Mini-PROTEAN II Dual Slab Cell (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were electrophoresed at 25 V onto a 0.22 μm nitrocellulose membrane overnight. After probing with anti-rabbit IgG horseradish peroxidase (HRP)-conjugated (Pierce Chemical Co., Rockford, IL, USA; 1:10,000) for 1 h, the intact and cleaved IgG molecules were detected by its reaction with Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.). Chemiluminescence generated was captured by a UVP-Chemi System (UVP, Inc., Upland, CA, USA). The amount of IgG was quantified by the LabWorks” Image Acquisition and Analysis Software (UVP, Inc., Upland, CA, USA). TR activity was determined as a percentage reduction of intact IgG.

Trx expression

The abundance of brain Trx was quantified by western blotting. Briefly, 200 μg brain homogenate was resolved by SDS-PAGE (12% separating gel and 4% stacking gel) and electro-blotted onto a nitrocellulose membrane. The membrane was first probed with mouse IgG raised against rat Trx (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:2000), followed by probing with anti-mouse IgG HRP-conjugated antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:1000) for 2 hours with gentle shaking. The detection and quantification of brain Trx were in accordance with the procedures abovementioned. The expression unit was defined as pg Trx per mg homogenate protein. Selected blots were re-probed with mouse IgG against rat α-tubulin as an internal control.

Statistical analysis

Data were expressed as mean ± standard deviation. Comparisons among different groups were made using multiple analyses of variance (ANOVA) followed by post-hoc protected least-significant difference test. In all instances, P < 0.05 was considered statistically significant.

Results

TR activity on DTNB reduction

In the presence of NADPH, TR activity was determined from the reduction of DTNB by brain homogenate obtained 1 day or 4 days after transient focal ischemia (Fig. 1). TR activity measured in the normal group (4.78 ± 1.09 mol NADPH oxidized per min per mg protein) was comparable to those in the ischemic Day 1 group (5.23 ± 0.65) and ischemic Day 4 group (5.185 ± 1.08). At either post-ischemia day 1 or day 4, statistical analysis revealed that there was no significant difference observed among all experimental groups. These observations suggested that TR activity on reducing small disulfide-containing molecules was not affected by the induction of cerebral ischemia or EA stimulation.
Fig. 1. Thioredoxin (TR) activity of right brains of normal and operated groups after 1-day or 4-day reperfusion following 1-hem CICO. Data represents mean ± S.D., n = 4 in each group. TR activity was determined from an initial increase of DTNB reduction as measured at 412 nm in 1.6 mM TBA, 0.1 M Tris-HCl (pH 7.13), 5 mM DTNB, 0.3 mM NADPH. Activity unit was defined as nanomoles NADPH oxidized per mm per mg protein. Statistical analysis revealed no observable significant differences.

TR activity on the reduction of IgG molecules

In the presence of both NADPH and Trx, TR activity on reducing protein disulfide bridges in IgG molecules was measured. The reduction pattern of the intact IgG molecules was obtained (Fig. 3A) and the percentage of IgG reduction was derived after quantifying the bands corresponding to intact and cleaved IgG molecules (Fig. 2B). The percentage of IgG reduction by TR obtained from the Normal group was 74% ± 0.76%. At post-ischemia Day 1, all experimental groups maintained a similar level of TR activity on IgG reduction comparable to the Normal group. In addition, there was no statistical significance between all experimental groups obtained 1 day after transient focal ischemia. At post-ischemia day 4, an only increase in the percentage of IgG reduction (78% ± 0.69%) was observed in the Ischemia Day 4-EA-ST36 (IS-3) group. Its TR activity was 10% (P < 0.05) higher than that of the Normal group and 14% (P < 0.05) higher than that of the Ischemia Day 4-EA-GB20 group. All remaining groups did not exhibit markedly altered TR activities on IgG reduction.

Trx expression

Another component of the thioredoxin system, Trx, was also detected. Its expression patterns obtained from western blotting was shown in Fig. 3A. The results of their corresponding amounts
Fig. 3. (A) Reduction pattern of IgG protein disulfide by brain TR of experimental groups after transient focal cerebral ischemia for 1 day or 4 days. Brain homogenate was incubated with 200 μg rabbit IgG, 3 μM PhenylmethyltrioxTrx and 50 μM NADPH in 20 mM Tris-HCl (pH 7.4). After incubation at 37°C for 30 min, all reaction mixtures were boiled and subjected to SDS-PAGE. Proteins in the gel were transferred onto a nitrocellulose membrane. It was then blocked with 5% skimmed milk, followed by conjugating with polyclonal anti-μAb IgG (H+L)-conjugate. Intact and cleaved IgG molecules were detected by means of reconstituted chromogenic reagents and then quantified. Treatment “C” represents the positive control where intact IgG molecules were reduced by p-methanesulfonic (B) Quantification of reduced IgG molecules. TR activity was determined as a percentage of IgG reduction. Data represents mean ± S.D., n = 6 in each group. Symbols (a, b, c) represent a significant difference compared to the Normal group (P < 0.05) and between experimental groups with EA stimulation at two subsequent points harvested on the same day (P < 0.05), respectively.
Fig. 3. (A) Trx expression pattern of normal and operated brain harvested after transient focal ischemia at post-ischemia day 1 and day 4. Brain homogenate (200 µg) was resolved by SDS-PAGE and electro-blotted onto a nitrocellulose membrane. It was then blocked with 5% skimmed milk, followed by conjugating with mouse monoclonal anti-rat Trx antibodies and red-raise IgG-HRP-conjugated secondary. Trx was detected by means of enhanced chemiluminescence and was captured and quantified. An internal control, α-tubulin, was selectively probed. (B) Its expression was quantified as pg Trx per mg protein. The data represents mean ± S.D., n = 6 in each group. Symbol (a) represents a significant difference compared to the Normal group (P < 0.05). On the same harvest day, symbols (b, c) denote significant differences to the Sham EA group (P < 0.05), and to the group with corresponding Sham EA stimulation (P < 0.05), respectively. Symbol (d) indicates a significant difference between groups with EA stimulation at a particular pair of acupuncture points. Harvested at day 1 and 4 (P < 0.05).
on the 1st or 4th day after transient focal cerebral ischemia (P < 0.05). In comparison to the ischemic group on day 1 or day 4, Trx expression remained unchanged in those groups with Sham EA stimulation at either Fengchi or Zusani.

However, some interesting findings were observed in groups with EA stimulation. For example, the amount of Trx in the ischemic Day 1 (I-A, GB20 (11-GB) group increased to 0.249 ± 0.016 ng Trx per mg protein, 10% higher than that of the ischemic Day 1 (II) group (P < 0.05). Moreover, after 4 days of transient focal cerebral ischemia, an upregulated Trx expression was observed in groups with EA stimulation at either Fengchi or Zusani. Their expressions were over 20% greater than those of the ischemic Day 4 (III) group (P < 0.05) and the groups with Sham EA stimulation at each corresponding acupuncture point (P < 0.05). Apart from the significance in the nature of treatment delivered to each group, statistical analysis revealed that Trx expression was also dependent on when harvest occurred after cerebral ischemia. Significantly induced Trx expressions were detected when comparing groups with EA stimulation at each acupuncture point harvested on either day 4 or day 1, i.e., 11-GB group against 11-GB group (P < 0.05) and 14-ST group against 11-ST group (P < 0.05). However, there was no observable difference between the 11-GB and 11-ST groups and between the 14-GB and 14-ST groups, indicating that EA stimulation at either acupuncture point may produce a similar effect on regulating Trx expression.

Discussion

In the present study, we first examined the disulfide-reducing activities of TR and the expression level of TR in brains after transient focal cerebral ischemia. TR activities on the NADPH-dependent reduction of small molecules (DTNB) remained relatively constant. Similar activity levels were measured on the Trx-mediated reduction of disulfide-containing proteins (IgG molecules). Our findings agreed with the observations that TR was not likely to be induced by various stresses (Takagi et al., 1998; Becker et al., 2000). On the other hand, as reported previously, Trx expression was known to be affected by stress (Nakamura et al., 1994; Obara et al., 1999). In our study, we found that the induced expression of Trx started as early as at 1 day and sustained at 4 days after cerebral ischemia-reperfusion injury. Takagi et al. (1998) found that Trx mRNA measured by Northern blot analysis was induced at 8 hours and increased until 24 hours of reversible MCAO. Their immunohistochemical studies also reported that neurons in layer II and V showed intense Trx immunoreactivity after 24 hours of transient MCAO (Takagi et al., 1998b). Similarly, Trx induction was regarded as an early event in focal trauma and motor nerve injury that had already occurred at 24 hours post injury (Lippoldt et al., 1995; Munson et al., 1998). However, a sustainable expression level of Trx has not been reported in cerebral ischemia-reperfusion injury. It is only in the motor nerve injury model that Munson et al. (1998) revealed that the upregulation reached its peak in about 7 days and then gradually decreased after more than 8 weeks. The induction of Trx expression was likely related to the generation of ROS because of the stronger expression of Trx mRNA and proteins in ischemia-reperfusion than in ischemia only (Takagi et al., 1998b). Taken together, we suggest that ROS-mediated induction of Trx expression was not transient and its induced levels could be maintained for a significant period after injury.
To the best of our knowledge, we are the first group examining the TR activities and Trx expression on ischemic rats with EA stimulation at either Fengchi (GB20) or Zusanli (ST36). As monitored in the reduction of DTNB or IgG molecules, TR activity was not altered when EA stimulation was applied on GB20. However, activity level of TR increased at post-ischemia day 4 after EA stimulation on ST36. This increased activity may work alongside the induced Trx expression to protect cells from protein disulfide oxidation produced by cerebral ischemia-reperfusion injury. On the other hand, Trx expression was more susceptible to EA stimulation on each acupoint. Both expression levels gradually increased from post-ischemia day 1 to day 4. In cerebral ischemia, a reduced form of glutathione (GSH) was being depleted by its reaction with acutely produced nitric oxide intermediates to form S-nitrosoglutathione (GSNO) (Burton, 1985; Gaston et al., 1993). The depleting level of GSH limited the activities of glutathione peroxidase (GPx) (Luthman and Holmgren, 1982; Takagi et al., 1988a), leading to its reduced ability to remove ROS intermediates. In our study, an over-expression of Trx by EA stimulation could likely restore the active GSH by reducing the amount of GSNO formed (Burton, 1985; Gaston et al., 1993). Additionally, Trx could replace GSH as the substrate for GPx (Nakamura et al., 1994; Lippold et al., 1995; Takagi et al., 1988a; Grimmer et al., 1999; Amér and Holmgren, 2000; Masatomo and Powers, 2000; Prolo et al., 2000). This dual action of Trx probably ensured that GPx stayed fully active in protecting the cells from injury by hydrogen peroxide. Induced levels of Trx could directly promote the expression of manganese superoxide dismutase (MnSOD), resulting in an increased removal of highly reactive superoxide anions (Colledge et al., 1996; Des et al., 1997; Nordberg and Amèr, 2001). A higher removal rate of superoxide anions can effectively protect the cells against nitric oxide injury where the cytotoxic reactive molecule, peroxynitrite, is formed (Des et al., 1997; Franklin and Brame, 1998). Such Trx-directed induction of MnSOD and GPx activity may be regarded as a compensatory increase in protective enzyme activity in response to increased oxidative stress (Lippold et al., 1995; Lou et al., 2000). Moreover, the application of electrical stimulation was necessary to produce the observed neuroprotective effect (Shi et al., 2004). Other clinical studies reported that a single application of EA stimulation on GB20, as opposed to manual acupuncture, was capable of improving blood circulation in cerebral arteriosclerosis (Yu et al., 1999), increasing anti-inflammatory enzyme activities in vascular dementia (Li et al., 1997), and restoring speech ability in stroke patients (Gao et al., 1995; Liu et al., 2000). In our experiment, neither TR activity nor Trx expression was modified in the ischemic rats with Shanzhao EA stimulation where no electrical stimulation was given to the inserted acupuncture needles. Our observations may further suggest that a single application of EA stimulation at bilateral ST36, not only GB20, produces neuroprotective effects on an animal stroke model.

Both acupoints are currently being used in clinical treatment for stroke patients, since EA produces beneficial effects such as improved motor functions (Jian, 1989; Xu and Cai, 1999; Liu et al., 1998; Su et al., 1998; Pei et al., 2001), regulated blood flow (Research School of Chinese Medicine, 1979; Yuan et al., 1992; Yuan et al., 1998), and reduced blood viscosity (Qi et al., 1997; Chen et al., 1998; Zhao et al., 1999). However, some clinical studies found that repeated EA stimulation at GB20 was more effective than at ST36 in increasing locomotion (Liu et al., 1999) and restoring speech ability (Zhang and Ni, 1998). In our study, we were unable to differentiate any difference by using a single EA stimulation at GB20 and ST36 after cerebral ischemia. The variation may be partly due to the number of treatment sessions of EA stimulation and also the conditions determined by our treatment protocol.
Conclusion

In conclusion, our study demonstrated that EA stimulation either at Fuguchi (GI820) or Zusanli (ST36) could upregulate the activity of Trx system by increasing the availability of Trx to TR and thereby reducing the ROS induced formation of disulfides.

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References


Treatments of Experimentally Induced Transient Cerebral Ischemia With Low Energy Laser Inhibits Nitric Oxide Synthase Activity and Up-Regulates the Expression of Transforming Growth Factor-Beta 1

Mason C.P. Leung, et al.

Background and Objectives: Nitric oxide (NO) has been shown to be neurotoxic while transforming growth factor-beta 1 (TGF-β1) is neuroprotective in the stroke model. The present study investigated the effects of low energy laser on nitric oxide synthase (NOS) and TGF-β1 activity after cerebral ischemia and reperfusion injury.

Study Design/Materials and Methods: Cerebral ischemia was induced for 1 hour in male adult Sprague-Dawley (S.D.) rats with unilateral occlusion of middle cerebral artery (MCAO). Low energy laser irradiation was then applied to the cerebrum at different durations (1, 5, or 10 minutes).

The activity of NOS and the expression of TGF-β1 were evaluated in groups with different durations of laser irradiation.

Results: After ischemia, the activity of NOS was gradually increased from day 5, became significantly higher from day 4 to 8 (P < 0.01), but returned to the normal level after day 7. The activity and expression of the three isoforms of NOS were significantly suppressed (P < 0.01) in different extents after laser irradiation. In addition, laser irradiation was shown to trigger the expression of TGF-β1 (P < 0.001).

Conclusions: Low energy laser could suppress the activity of NOS and up-regulate the expression of TGF-β1 after stroke in rats.

Key words: laser; nitric oxide; stroke; transforming growth factor

INTRODUCTION

Nitric oxide (NO), a well-known cytotoxin, is known to be induced in permanent and transient cerebral ischemia (1,2). Overproduction of NO may be detrimental to neuronal survival in ischemia (3-5) and reperfusion injury (6,7). However, intracerebral administration of NO donors leads to an increase in blood flow and a decrease in infarct size in the focal ischemia model (8). The dual role of NO depends on the stage of ischemia insult. NO production is protective only during the very early stage (< 2 hours) of cerebrovascular ischemia (9,10) but becomes neurotoxic after a few hours (11).

Clinical findings suggest that increased NO generation is predominantly associated with cytotoxic effects (11). Transforming growth factor-β1 (TGF-β1) has been regarded as a protective endogenous mediator in cerebral ischemia and reperfusion (12). Administration of TGF-β1 could attenuate ischemic brain injury in vivo (13,14) and in vitro (15-17). The protective role of TGF-β1 may be related to the promotion of neuronal survival and neurite growth, regulation of fibroblast cell, and inhibition of astroglial cell division (18).

Low energy laser irradiation has been reported to reduce the infarct size after ischemia and reperfusion injury in the rat heart (19). Inhibition of NO production (20,21) and up-regulating the level of TGF-β1 (22) reduce infarct size in cerebral ischemia and reperfusion injury. We are interested to find out if low energy laser irradiation could alleviate level of brain injury by either decreasing the neurotoxic agents such as NO or increasing neuroprotective agents such as level of TGF-β1. To the best of our knowledge, this is the first report to show the effects of low energy laser on the expression of nitric oxide synthase (NOS) and TGF-β1 after cerebral ischemia and reperfusion injury.

MATERIALS AND METHODS

Induction of Cerebral Ischemia and Reperfusion

A total of 14 male Sprague-Dawley (S.D.) rats, weighing from 350 to 350 g, were used in this study. The rats were housed under standard conditions with food and water made available ad libitum. They were anesthetized by

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intraperitoneal (i.p.) injection of 70 mg/kg ketamine (Mallinckrodt) and 7 mg/kg xylazine (Alphax). During the surgical procedure, the rectal temperature of rats was monitored and maintained at ~37°C with an overhead lamp. The right middle cerebral artery (MCA) was excluded as previously described [25, 26] in which a reproducible infarct was obtained. Briefly, a scalp incision was made at the midpoint between the right eye and the right ear. The temporal muscle was separated in the plane of its fiber bundles and retracted to expose the bone and subcutaneous tissue. A 6 mm × 6 mm Burr hole was made 

with nitric oxide releasing substrate. The homogenate was centrifuged at 10,000 rpm for 20 minutes at room temperature. The supernatant was then treated with 100°C for 10 minutes. The sample was centrifuged and the supernatant was transferred to a new tube. The protein was assayed using the bicinchoninic acid method. The protein concentration was determined using the BCA protein assay kit (Pierce). The samples were then boiled for 5 minutes and the absorbance was measured at 562 nm using a microplate reader. The protein concentration of the samples was determined as nanograms of NOS per micromoles of protein.

Expression Study of TGF-β1 by Western Blotting

SDS-PAGE and electrophoretic transfer of protein was performed as described in the previous section. After blotting, the blots were blocked with rabbit anti-rat TGF-β1 IgG (Santa Cruz Biotechnology, Inc. at 1:1,000 overnight at 4°C). The blots were washed in TBS-T for 5 minutes each at room temperature. The blots were then incubated in 1:1,000 NBS per micromoles of protein.

Expression Study of TGF-β1 by Western Blotting

SDS-PAGE and electrophoretic transfer of protein was performed as described in the previous section. After blotting, the blots were blocked with rabbit anti-rat TGF-β1 IgG (Santa Cruz Biotechnology, Inc.) at 1:1,000 overnight at 4°C. The blots were washed in TBS-T for 5 minutes each at room temperature.
LASER EFFECTS ON CEREBRAL ISCHEMIA

1 mg/kg Intravenous pentoxyfylline-conjugated goat anti-rabbit IgG antibody (Pierce Chemical Co.) for 2 hours at room temperature with gentle shaking. Three times were used as an antiserum with TBS for 3 minutes each at room temperature. The amount of TGF-β1 present was detected by blocking the binding with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.). This chemiluminescence was captured and analyzed by UVP Chemi-Sys (UVP, Inc.). The expression of TGF-β1 was defined as nanograms of TGF-β1 per micrograms of protein.

Time Dependent Response of Specific Activity of NOS After Cerebral Ischemia and Reperfusion

Serial expression of NOS was followed after the cerebral ischemia and reperfusion. Animals were used to detect the specific activity of NOS from post-injury day 1-3 and day 8. Sample size of the normal group was 11, while the ischemic groups were eight for each time point.

Laser Irradiation

Immediately after MCAO, a laser probe (gallium aluminum arsenide) with a divergence of 10 cone (spot area = 20 mm² at 5 mm distance) was directed to a laser beam at 630 nm, average power of 0.6 W, energy density at 1 minute with 2.66 J/cm² and pulse frequency of 10 kHz (Omega Excel Laser, London, United Kingdom) was applied directly through a burr hole 5 mm from the cerebrum immediately. The laser was applied for 5, 10, or 10 minutes (Lasers, n=4, Lasers, n=4, Lasers, n=4, Lasers, n=4, respectively). The control group (whole homogenate samples obtained from the ischemic group at post-injury day 4 in the time-dependent response studies) received the same surgical procedure but was not exposed to laser. The normal group (whole homogenate samples obtained from the normal group in the time-dependent response studies) was subjected to neither surgery nor laser. According to the serial study on the specific NOS activity after cerebral ischemia and reperfusion, the maximum NOS activity was found at post-injury day 4. Therefore, animals in this experiment were sacrificed at post-injury day 4 for detecting the specific activity of NOS and the amounts of NOS forms and TGF-β1.

Statistical Analysis

Expression levels of enzymes measured are expressed as mean ± S.D. Statistical differences between means were determined by ANOVA followed by a LSD test. A probability value of < 0.05 was accepted as significant for differences between groups of data. All the statistical procedures were performed using SPSS version 11.0. SPSS Inc., Chicago, Ill.

RESULTS

Serial Expression of Specific Activity of NOS In the Brain After Cerebral Ischemia and Reperfusion

After cerebral ischemia and reperfusion, the total specific activity of NOS in the cerebral cortex was increased slightly in post-injury day 3, significantly from post-injury day 4 to 5.0 (P < 0.001, ANOVA followed by LSD test) and then back to normal level at post-injury day 7 (Fig. 1). As the peak level was found at post-injury day 4 (52% more than normal), therefore, we decided to study the effects of laser at this peak MCAs point.

Effects of Laser on NOS

The total functional specific activity of NOS was significantly decreased (P < 0.001, ANOVA followed by LSD test) after different durations of laser irradiation at post-injury day 4 compared with the control group (Fig. 2). This decrease was more obvious with longer laser irradiation (Laser 10 min, n=4). The group had 58% (P < 0.001, ANOVA followed by LSD test) and 75% (P < 0.001, ANOVA followed by LSD test), respectively, when compared with control group which Laser 10 min had 65% (P < 0.001, ANOVA followed by LSD test) of NOS activity.

Expression of the three NOS forms (Fig. 3) had similar trends with the specific activities of NOS. Expression of the three NOS forms was significantly down-regulated after different durations of laser irradiation (P < 0.001, ANOVA followed by LSD test). The extent of decrease was highest in eNOS followed by iNOS, and least in NOX. For example, expression of iNOS in the Laser 10 min groups dropped by 35%, 48%, and 38% for eNOS, iNOS, and NOX, respectively. The results indicated that low energy laser irradiation could suppress the expression and activity of NOS in the post-cerebral ischemia and reperfusion model.

Effects of Laser on TGF-β1

TGF-β1 level could not be detected in the brains of normal and control groups. However, after different lengths of laser irradiation, TGF-β1 expression in the right hemisphere was up-regulated significantly (P < 0.001, ANOVA followed by LSD test) (Fig. 4). There was no significant difference among the three laser groups. Nevertheless, this indicated that all three durations of laser irradiation were effective in up-regulating the expression of TGF-β1 after cerebral ischemia and reperfusion.

![Graph showing expression levels of specific activity of NOS and TGF-β1](image)

Fig. 1. Serial expression levels of specific activity of NOS (nanograms of nitric oxide produced per hour per milligrams of protein) after cerebral ischemia and reperfusion. The activity was increased significantly from day 3 to day 8 and then back to normal level. Data are mean ± S.D. *P < 0.001 vs. normal, LSD.
**DISCUSSION**

Nitric oxide enhances oxidative insult by reacting with superoxide anion to form a stronger oxidant, peroxynitrite, and perturbing iron metabolism [28-29]. It leads to mitochondrial dysfunction, genome DNA damage and triggers apoptosis [30,31]. Apoptic neuronal death mainly contributes to infarct formation in cerebral ischemia [32-34].

The activity of NOS in this right hippocampus was gradually increased from post-injury day 3, then sustained for 3 days, and dropped back to normal level. A few days sustained increase of NO levels may be due to the expression of iNOS within microglia, invading inflammatory cells, and cerebral vascular endothelium after MCAO [35,46]. Our results indicate that the increased expression was not only confined to iNOS but also present in eNOS and nNOS. Both nNOS and iNOS expression were obviously up-regulated as post-injury day 4. However, a continuous depression of constitutive NOS activity was reported up to 10 days after ischemia [37]. It is possible that regional cerebral blood flow (rCBF) dropped to 9% of the baseline level but maintained enhancement in NO synthesis in areas where rCBF was 16% [38]. Thus, in our studies, using the model of transient ischemia and reperfusion injury, the up-regulation of NOS activity is expected.

Furthermore, nNOS knockouted developed smaller infarct and reduced neurological deficits than the wild-type mice after 3-4 days after MCAO [39]. Neuroprotection can be achieved by early administration of selective iNOS inhibitors in mice [40]. Therefore, it is inferred that inhibition of NOS activity reduces cell death [41]. In our study, when the low energy laser irradiation, both the activity and expression of NOS were suppressed and it may be important in cell survival. It has been shown that Helium Neon laser irradiation could minimize inflammation by inhibiting the enhanced production of proinflammatory cytokines and leukotrienes [43]. Whether the suppression of NOS we have seen in our study follows the same pathway is currently unknown.

It is interesting to find that, when using the ELISA method, TGF-β1 is normally not detectable in serum samples from normal individuals [43] but is found in stroke patients [43]. In our studies, both normal and control groups had no detectable levels of TGF-β1. TGF-β1 is possible that ELISA (protein level) is more sensitive than Western blot (mRNA level) methods. However, after laser irradiation, TGF-β1 became detectable with three different durations of irradiation. TGF-β1 has been shown to exhibit anti-inflammatory properties [44,45]. It suppresses the expression of iNOS [46,47], inhibits mononuclear cell activities [48], and reduces infarct size in stroke model [49,50]. Laser irradiation may be neuroprotective by up-regulating TGF-β1 and suppressing NOS.

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Fig. 2. Effects of different durations (1, 5, or 10 minutes of low energy laser irradiation on the specific activity of NOS (nanomoles of nitrate produced per hour per milligram of protein) after cerebral ischemia and reperfusion at post-injury day 4. The activity was measured after laser irradiation. Data are mean ± SD (*P < 0.001 vs. control, ANOVA followed by LSD test). NOX, constitutive NOS; iNOS, inducible NOS.

Fig. 3. Effects of different durations (1, 5, or 10 minutes of low energy laser irradiation on the expression of three isoenzymes of NOS (nanograms of NOS detected per microgram of protein) after cerebral ischemia and reperfusion at post-injury day 4. The expression of NOS was measured after laser irradiation. Data are mean ± SD (*P < 0.001 vs. control, ANOVA followed by LSD test). NOX, constitutive NOS; iNOS, inducible NOS.

Fig. 4. Effects of different durations (1, 5, or 10 minutes of low energy laser irradiation on the expression of TGF-β1 (nanograms of TGF-β1 detected per microgram of protein) after cerebral ischemia and reperfusion at post-injury day 4. The expression of TGF-β1 was up-regulated after laser irradiation. Data are mean ± SD (*P < 0.001 vs. control, ANOVA followed by LSD test).
Experiments have shown that low energy laser irradiation delays degeneration of peripheral or central nerve terminals (46, 50) and electrophysiologically in animals (43, 51). Other studies demonstrated that low energy laser irradiation applied to the brain tissue of rats might modulate procollagen synthesis (43, 51) and increase the amounts of procollagen and procollagen in rats subjected to cerebral ischemia (66, 86), thereby improving neurotrophic function. SOD is one of the first cellular responses to oxidative stress and scavenges the highly reactive superoxide radical and hydrogen peroxide, thus preventing the formation of dangerous substances. This study was conducted to investigate the effect of low energy laser irradiation on neurotrophic function.

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Total Retinal Nitric Oxide Production is Increased in Intraocular Pressure-elevated Rats

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Nitric oxide (NO) is a well-known vasodilator that has no regulation in the retina is unclear. This study was conducted to quantify total NO production and retinal ganglion cell (RGC) loss in an experimental glaucoma model. Three quarters of the peribulbar/retinal damage were then treated with a single injection (100ug) of both eyes using a single-tissue transplantation technique. At days 14, 28 and 35 after the laser treatment. After 15 days, we determined the total NO level in retinas and remaining order lenticular for the laser-treated and control eyes using a spectrophotometric assay. The viable RGC number was also determined by counting the cell bodies stained en face immunocytochemically with iboxojid. The laser treated significantly increased the IOP 2.0-2.4kPa throughout the whole period of measurements (P<0.001). The mean total RGC number decreased significantly from 72%±5% to 33%±8% at 28 days and 27%±5% at 35 days, while IOP remain unchanged. The mouse total NO level in the laser-treated retina was significantly increased by 2.4kPa compared with controls (P<0.01) but no significant difference was found in the cytosol P<0.01). Laser treatment resulted in significant IOP elevation and RGC loss, suggesting that thermal degeneration of the peribulbar region may provide an alternate protocol for glaucoma study. NO level was increased by two-fold in the retina but not in other ocular tissues. Since NO is capable of producing powerful peroxynitrite anions, and nitric oxide derived nitrotyrosine against elevated IOP-induced ganglion cell damage, suggesting a potential role of excitation in retinal toxicity.

1. Introduction

Glaucoma is a sight-threatening eye disease. It is characterized by an elevated intraocular pressure (IOP), optic neuropathy and visual field loss (Humphrey et al., 1984). Glaucoma is the leading cause of vision loss and affects 1.9% of the world population and causes primary open-angle glaucoma, with one-third of these proceeding to bilateral blindness (Humphrey, 1975). Because of its insidious nature, many sufferers were frequently unaware of the condition until very late stage.

Although the detailed pathogenesis remains uncertain, elevated IOP is clinically associated with glaucomas by damaging the larger retinal ganglion cells (Nguyen et al., 1999). Direct mechanical pressure on indirect mechanism may account for the damage (Kassam, 1984). However, Ko et al. (2000) have recently demonstrated that an anti-inflammatory agent complements the protective effect of the brain-derived neurotrophic factor against elevated IOP-induced ganglion cell damage, suggesting a potential role of excitotoxicity in retinal toxicity.

Nitric oxide (NO) is a messenger molecule that mediates neurotransmission (Dawson and Snyder, 1994) and vasodilation (Hardy et al., 1998). In retinas, NO is produced by groups of neurons called retinal oxide synthase (NOS) (Cao et al., 1999; Neufeld et al., 2000). In the eye, NO regulates the basal ocular blood flow rate in response to physiological stress (Lee, 1999) and suppression of NO production reduces the choroidal blood flow (Schmidt et al., 1997). A number of studies have indicated that NO may be involved in the pathogenesis of glaucoma (Dawson and Snyder, 1994). NO has been shown to act as a neuroprotective agent in the retina (Schmidt et al., 1997). However, a direct link between retinal NO level and IOP elevation is yet to be established. Since NO has an unpaired electron in the outer orbit, its inherent free radical nature may impose excitotoxicity on the retina (Norris et al., 1997). This study aimed to quantify the NO production in an experimental glaucoma rat model.
2. Materials and Methods

Intra-canal Pressure-elevated Rat Model

The right eye of 12 Sprague-Dawley rats (body weight approximately 300 g) received laser irradiation at the limbal region as proposed by Wadle-Maxwell et al. (1995) and others (Sorensen et al. 2001; Schaefer et al. 2001; Leopold et al. 2002). The laser equipment (Ultima 2000se Argon Laser, Coherent, Santa Clara, CA, USA) was attached to a slit lamp (Haag-Streit 900). The treatment blocked the episcleral and limbal drainage vessels and surrounding connective tissue in a 270° arc around the perilimbal region (except the nasal quadrant) to produce a sufficient outflow blockage. The animals received two laser treatments on two separate days (7 days apart) under general anesthesia (intraperitoneal injection of 7.5 mg kg⁻¹ ketamine and 7 mg kg⁻¹ xylazine mixture). Both corneas were further anesthetized with a topical application of 0.5% proparacaine (Alcaine; Alcon, Fort Worth, TX, USA) eyedrops. Approximately 1.5 and 3.0 laser irradiations (100 mW; spot size 50–100 mm) were applied to the episcleral areas and the limbal veins, respectively.

After the treatment, the animals were returned to the cage with a topical application of 0.5% tribecaine (Thibrex; Alcon, Paris, Belgium) on both eyes. Food and water were provided 24 h before and the animals were kept in a temperature-controlled room (12 hr light/12 hr dark cycle).

NIT Measurements

The NIT of four animals were measured using a digital tonometer (Medtronic Tonopen XL, Solan Optik, Jacksonville, FL, USA) under the general and local anesthesia described earlier. Seven readings were recorded from each eye and the average value was taken. IOP readings were taken on days 21, 28, and 35 after the completion of the second laser treatment. All measurements were taken between 10:00 and 11:00 hr.

Retinal Ganglion Cell Number

Twenty-eight days after the second laser treatment (7 days before killing), head of the animal was anesthesia stereotactically under general anesthesia (70 mg kg⁻¹ ketamine and 7 mg kg⁻¹ xylazine mixture). Two small holes (2 x 2 mm) were made on the skull bilaterally above the superior colliculi (SC) after the induction of skin. Cortical tissues above SC were removed using an aspirator. Gilford (Hopkins, Kalamasoo, MI, USA) inked in 0.5% fluorescein (G5; Fluoresceine Dye; Co. USA) were applied onto both surfaces of SC for these four animals and then the skin was sutured. The animals were returned to the cages after the procedure. Fix was then up by the agent of RGF and transported retrogradely to the cell bodies in the retina. Seven days after the FG treatment (i.e., Day 35), the animals were killed with an overdose of the anesthetics in saline and the retinas were removed. The retinas were soaked in 4% paraformaldehyde phosphate buffer solution for 1 hr and flat-mounted on microscopic slides.

The retinas were divided into four quadrants. Labelled RGCs were counted along the medial line of each quadrant in 0.5 mm steps from the optic disc (magnification 400 x) using a computerized imaging software (Neuronalux Wilotec, VT, USA) and a fluorescent microscope (Nikon, Kowasaki, Japan). About 30–32 microscopic fields of 145 x 127 mm² were counted. The total numbers of FG-labelled RGCs were determined per retina.

Nitric Oxide Production

The retinas of another eight animals were collected from the eyeballs of a saline bath 35 days after the second laser treatment. The remaining corneal tissues (i.e., cornea, sclera, iris, vitreous, ciliary body and vitreous body) were also collected and pooled together.

The samples were homogenized in 150 μl ice-cold Tris buffer solution (20 mm, pH 7.4) and 40 μl protease inhibitor cocktails (Sigma St. Louis, MO, USA) using a mortar and pestle in an ice-cold (0°C) environment. After the centrifugation (15 750 g) for 20 min, the supernatants were collected and incubated with excess L-arginine, free formyl donors (NADPH, BH₄, FAD, FMN, and calmodulin) and CuCl₂ at 37°C for 4 hr. The substances are essential elements for the NOS to produce NO. Another set of supernatant was also prepared without any incubation (served as reference). Five and six valid sets of data were obtained for the laser-treated and control retina, respectively.

NO quickly converts into nitrate and nitrite in the biological system. The amounts of total nitrate/nitrite were determined using a colorimetric assay kit (Cayman, USA). The samples developed purple color after the addition of Griess Reagents (sulfanilamide and N-ethylmaleimide). Briefly, the nitrate was first converted into nitrite by adducing nitrate reductase. The acidified nitrite combined with sulfanilamide that further reacted with N-ethylmaleimide to form a stable Az product. The absorption of the sample mixtures at 540 nm were measured. The nitrate/nitrite amounts were determined by comparing the photometric values with and without the incubation.

The amount of protein was also determined using the Bradford (Biorad, Hercules, CA, USA) assay. The photometric absorption at 595 nm was checked against a calibration curve derived from the bovine serum albumin standards. The total amount of nitrate/nitrite per unit weight protein provided an index of NOS activities in the tissues.
3. Results

Intraocular Pressure

The mean IOP values in the laser-treated eyes were higher than those of the controls over the period studied (Fig. 1). The difference in IOP between treatment and control eyes was statistically significant (two-way repeated measures ANOVA: F = 101.5, P < 0.0001). There was a statistically significant difference in the IOP values obtained on different days (two-way repeated measures ANOVA: F = 5.68, P < 0.0025). Post-hoc test results showed that the difference in IOP between the treated and untreated eyes was statistically significant for all 3 days of measurements (Bonferroni's multiple comparisons, P < 0.01).

There was no statistically significant interaction between the effect of laser treatment and day of measurement (two-way repeated measures ANOVA: F = 1.25, P = 0.33).

On day 21, the mean IOP values in the control and laser-treated eyes were 9.1 and 9.6 mmHg, respectively. The mean IOP value of the laser-treated eyes was approximately 2.6 times higher than the control eyes. Similarly, there was a 2.4 and 2.2 times difference in IOP between the laser-treated and control eyes measured on day 28 and 35, respectively. Post-hoc testing revealed a significant difference in the mean IOP between day 21 (9.1 mmHg) and 28 (11.4 mmHg) for the control eyes (Bonferroni post test; P < 0.01) but the difference between the laser-treated eyes was not statistically significant for any 2 days (Bonferroni post test; P > 0.045).

Retinal Ganglion Cell Number

Five weeks after the laser treatment, the average total RGC number of the control eye was 98,723 ± 5,813 (±SEM, n = 13). The average total RGC number of the laser-treated eye decreased significantly to 40,276 ± 5,992 (t-test: t = 6.227, P = 0.0008). This corresponded to a 59.8% reduction in the RGC (Fig. 2).

Nitric Oxide Production

The total levels of nitrate/nitrite were different after the laser treatment (Fig. 3). The difference was statistically significant (two-way ANOVA: F = 6.74, P = 0.016). There was a statistically significant difference in total nitrate/nitrite levels between the combined ocular tissues and the retina (two-way ANOVA: F = 4.170, P = 0.0494). The retinal NO level became lower 2.4 times compared with the control value after the laser treatment. Statistical analysis also revealed an interaction effect between the laser treatment and ocular tissues (two-way ANOVA: F = 7.70, P = 0.011). The laser treatment affected the retinal and ocular tissues' nitrate/nitrite levels differently.

![Fig. 1. IOP of the rats (mean ± SEM, n = 4)](image1)

![Fig. 2. The total RGC numbers mean ± SEM and n = 13)](image2)

![Fig. 3. The NO levels (mean ± 1 SEM) of the retina for the laser-treated (n = 5) and the control (n = 6) eyes. The data of the combined ocular tissues (eyesup) are also included (mean ± 1 SEM, n = 6).)](image3)
4. Discussion

The baseline IOP values obtained in this study were comparable to those reported in previous studies (Neufeld et al. 1999; Ko et al. 2000). The IOP values showed a statistically significant variation of 3.3 mmHg in the control eyes between days 21 and 28. The difference may be attributed to the natural fluctuations in blood pressure and the use of systemic antihypertensive medications. However, a consistent IOP elevation from approximately 17 to 18 mmHg was observed in the first 6 months. Using the same surgical procedures, Ko et al. (2000) reported a chronic two-fold elevation in IOP (from 12.9 to 25.4 mmHg) within the first 6 months. The present study employed a laser iridotomy, and this technique resulted in a 2.0-2.6 times increase in the treated eyes compared with the control eyes (17.9-23.6 mmHg). Similarly, Works et al. (2000) reported a two-fold IOP increase in eyes within the first 2 months after the laser treatment. The data confirm that bilateral surgical intervention produces a consistent elevation in IOP. The current animal model may provide an alternative experimental tool for studying glaucoma.

Thirty days after the laser treatment, the total RGC number was decreased by an average of 29.8%. The value is comparable to that of an earlier study in which 27.5% of the RGC were lost 33 days post-treatment (Ko et al. 2000). Neufeld et al. (1999) reported a further RGC loss of 35.9% after 6 months. These data suggest that chronic IOP elevation triggers a time-dependent RGC loss that is similar to human glaucoma (Shimazaki et al. 1999; Kerrigan et al. 1997).

Increased NO production has been reported in systemic sclerosis (Kaul et al. 2000), systemic lupus erythematosus (Wunnicke et al. 1998) and systemic lupus erythematosus (Mavrantzas et al. 2000). These diseases adversely affect the function of vital organs with substantial vascular circulation, e.g., kidney and lung. The retina, similar to these organs, receives ample blood supply from the choroid (Matsuo et al. 1992). Since the retinal blood flow is decreased under elevated IOP (Chung et al. 1999), it is possible that elevated pressure triggered NO activity, which is useful to restore normal vascular supply. Furthermore, Neufeld et al. (1999) showed that the application of aminoguanidine (NO synthase inhibitor) reduced the RGC damage in IOP-elevated retina to less than 10%. On the other hand, without the aminoguanidine treatment, the RGC loss was 35.9% after 6 months. These data suggest that NO might have a protective role in the retina. The present study showed that a two-fold elevation of IOP was accompanied by a significantly higher NO production in the retina, but not in other ocular tissues. The concurrent increase in NO production and RGC death is consistent with the notion that NO may be a physiological mediator in retina in response to elevated-IOP stress.

NO has diverse effects on living cells (Davson and Zetter, 1997). Selective inhibition of NOS caused no detectable effect in neuronal protection studies. For example, introduction of NO-synthase-arginine (s-NOS) to a retinal ischemic animal model demonstrated both lowering (Hansel et al., 1999) and protective (Lam and Lin, 1996) effects. Small amount of NO production is beneficial to the retina as it can up-regulate the blood circulation and thus facilitates the flow of metabolites. Excessive NO, however, may damage the retinal tissue (Oku et al., 1997) by a free radical oxidative mechanism (Blockman et al., 1994). NO is a free radical capable of causing cell death (Murphy, 1998). When NO combines with a superoxide anion (O$_2^-$), they form a potent and highly reactive peroxynitrite anion (ONOO$^-$). ONOO$^-$ may break down into hydroxyl free radical (OH$^-$) causing oxidative damage (Su et al., 1999) and the generation of other free radicals in a vicious cycle (Osathanondh et al., 1999). The chain reaction of free radical propagation may account for the three-dependent nature of many age-related diseases (Hansel, 1992). Since the total NO level in the retina was consistently higher than that of combined aqueous tissues in both the control and IOP-elevated eyes, the retina may be subjected to a significant risk of oxidative damage (Su et al., 1999).

Chronic overproduction of NO has been associated with neuronal degeneration, such as Parkinson's disease and Alzheimer's disease (Moncada et al., 1991). The retina is an extension of the central nervous system and it has a high polyunsaturated fatty acids (PUFA) content (Anderson and Maude, 1970) which is susceptible to oxidative damage. NO can either attack the double bonds on the membrane PUFA (Anderson et al., 1983) or modify the genetic nucleic materials (Murphy, 1999), causing necrotic and apoptotic cell death. The detailed mechanism of how the retinal NO activity is regulated under elevated IOP remains unclear. Further studies are required to elucidate the retinal NO regulatory mechanism under elevated IOP through different NOS sub-grouping.

As far as the oxidative behavior of NO is concerned, inhibiting NO production by NOS inhibitor (Neufeld et al., 1999) may provide neuroprotection to the retina by controlling the oxidative stress. However, the concurrent down-regulation of the retinal form of nicotinic cholinergic receptors may compromise the retinal blood circulation (Osathanondh et al., 1999), leading to further retinal damage in glucocorticoid eyes. Before the roles of NO are fully characterized, the application of NOS inhibitor in glucocorticoid (Nathanson, 1992; Krau, 1999) remains experimental. Meanwhile, preventive strategy (such as anti-oxidative supplement) may complement the stringent control of IOP to combat the glucocor-
related oxidative stress. The potential applications of antioxidants in retinal therapy deserve further attention.

In summary, the present study showed that light-induced blockage of epidermal and limb drainagewells caused a sustained two-fold IOP elevation, 29-8% RGC death and 2-4 tums NO production in the retina after 4 weeks. The results suggest that increased NO production may give a clue in glaucoma. The chronic optic hypertensive in rats after peripheral laser coagulation has resulted in significant RGC death and this may be a good animal model for glaucoma study.

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References


Regulation of caspase activation in axonotomized retinal ganglion cells

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Introduction

Axotomy of the optic nerve results in the death of retinal ganglion cells (RGCs). Interestingly, despite the severity of the injury, RGC loss was not observed until several days after axotomy. The mechanisms responsible for the initial lack of RGC death remained unknown. In the current study, immunohistochemical analysis revealed that caspase-3 and -9 activation in the RGCs were not detected until day 3 post-injury, coinciding with the onset of axotomy-induced RGC loss. Interestingly, elevated Akt phosphorylation was observed in axonotomized retinas during the absence of caspase activation, inhibiting the increase in Akt phosphorylation by intravitreal injection of Wortmannin and LY294002, inhibitors of PI3K, resulted in premature nuclear fragmentation, caspase-3 and -9 activity and apoptosis in the ganglion cell layer. Our findings thus indicate that the PI3K/Akt pathway may serve as an endogenous regulator of caspase activation in axonotomized RGCs, thereby, contributing to the late onset of RGC death following axotomy. © 2004 Elsevier Inc. All rights reserved.

Sectionally, morphological and biochemical analyses suggest that axonotomized RGCs die via an apoptotic pathway. Western blot analyses demonstrated that caspase-3 and -9 are activated in axonotomized rat retinas (Kernert et al., 1999, 2000a). In addition, intravitreal injections of caspase-3 and -9-specific inhibitors significantly enhance RGC survival at 14 days (Chandhary et al., 1999; Kernert et al., 1998, 2000a), indicating that the activation of these two caspases is involved in the demise of the damaged neurons. This suggests that the kinetics of caspase activation, and pathways participating in the regulation of caspase activation, may affect the rate of axonotomized RGC death. Therefore, the aim of the current study is to elucidate the molecular events constituting the late onset of detectable axonotomized RGC death by examining the kinetics of caspase activation, and the mechanisms implicated in the regulation of caspase activation.

In this study, we found that axonotomized RGC death commenced at 3 days in adult hamster. Intriguingly, activation of caspase-3 and -9 in axonotomized RGCs coincided with the late detection of axonotomized RGC death and was similarly noted observed until 5 days. The concurrent onset of caspase activation and RGC death indicates that the late activation of caspases may contribute the initial lack of RGC death following axotomy. We thus went on to examine the mechanisms of this delayed activation of caspases and if the delayed caspase activation contributed to the late death of axonotomized RGC death. Recent reports indicate that activation of the PI3K/Akt pathway serves as an important survival signals (Barber et al., 2001; Druéy et al., 1997; Nemer and de la Pompa, 1998; Pollitt et al., 2001). Akt is activated following phosphorylation at Serine 473 and Thrweine 328 after PI3K activation (O’Connor and Cotter, 2001). Akt activated Akt then exerts its anti-apoptotic effect by phosphorylating multiple targets downstream. Akt has been demonstrated to inhibit Bax to limit cytochrome c release (Dutta et al., 1997), in addition to displaying inhibitory effect on activated caspase-9 (Caronni et al., 1998; Piao et al., 2000). The PI3K/Akt pathway therefore provides a potential candidate for regulating caspase activation in axonotomized RGCs. In the current study, we examined the kinetics of Akt activation following axotomy. In addition, Akt phosphorylation in axonotomized retinas was reduced by injection of wortmannin, a PI3K inhibitor, to elucidate the functional significance of Akt activation in axonotomized retinas.

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Results

Cell death profile of ablated RGCs in hamsters

The number of surviving RGCs at 2, 3, 5, 7, 10, and 14 days was determined from flat-mount and retinal sections. The number of labeled RGCs at 1 day was not counted because the dye had not sufficiently reached the RGC soma, which might result in underestimation of the surviving RGCs. Surviving fluorescent (FG)-labeled RGCs were recognized by the fine, granular, and even distribution of FG staining. Cells exhibiting staining of FG in the ganglion, in cells containing potential photoreceptor vacuoles represented by tissue-localized vacuoles filled with FG were excluded. These are nonphotoreceptors attached to retinal cells that have engulfed degenerated RGCs (Rabbits and Wanger, 1958; Thomas et al., 1944). Fig. 1A shows the morphology of the surviving RGCs and the potential photoreceptor cells that were excluded from the RGC counts.

Several flat mounts have been used to quantify the number of RGCs following ablation (Kabler et al., 1994; Chu et al., 1998; Villesus-Perez et al., 1995). In the present study, sampling using retinal flat-mounts revealed that RGC loss was already observed at 3 days. If we assume the number of surviving RGCs at 2 days to represent the number of FG-labeled RGCs in undamaged retina, 38.3% of the total RGCs were lost within 3–5 days, representing the fastest RGCs have processes toward axon formation (Kabler et al., 1994; Chu et al., 1998; Villesus-Perez et al., 1995). In the present study, sampling using retinal flat-mounts revealed that RGC loss was already observed at 3 days. The number of cells with fragmented nuclei decreased thereafter, with continuous decrease in the number of surviving caspase-1- and -9-positive cells (Fig. 1A). On the other hand, activated caspase-3 and -9 immunoreactivities were found exclusively in the GCL. Of the activated caspase-3-positive cells, over 90% displayed fragmented nuclei and over 80% were FG-positive at all time-points (Figs. 2B and C). All of the activated caspase-8-positive cells displayed nuclear fragmentation and over 80% of these contained FG-labeling (Figs. 2D and E). These observations suggest that most of the cells exhibiting caspase activation were axonemal RGCs. Staining of retinal sections with FG labeling provided similar results, suggesting that FG-labeling did not interfere with the immunohistochemical staining. Our data indicate that activation of caspases and nuclear fragmentation was delayed by 3 days after ablation, coinciding with the onset of axonemal RGC death.

The activated caspase-3 immunoreactivity was caspase-9-dependent

To elucidate the causal relationship between the activation of caspase-3 and -9 activation observed following ablation, we investigated if the activated caspase-3 immunoreactivity was dependent on caspase-9 activation. LEHD-cho and LEHD-cho, the nonsubstrate and non-substrate-caspase-3 inhibitors, function as competitive substrates for the activated enzyme to inhibit its activity (Guilastra et al., 1998; Tomberlin et al., 1997). Inhibitory effects of LEHD-cho and LEHD-cho have been shown to significantly reduce caspase-9 activity at 3 days in the axonemal RGCs (Schwarz et al., 2000a), suggesting that the dosage adopted could effectively inhibit cas-
Fig. 2. Immunohistochemical analysis of nuclear fragmentation, caspase-3 and caspase-9 activation following injury. (A) The number of cells displaying fragmented nuclei, both nuclear fragmentation and activated caspase-9 immunoreactivity or both nuclear fragmentation and activated caspase-9 immunoreactivity, was increased in experimental animals compared to sham-operated animals at 1 and 2 days after injury. The number of activated caspase-3, caspase-9 activation and nuclear fragmentation was increased at 3 days, and decreased thereafter. (B and C) Fluorescence photomicrographs of the same animal section stained with antiactivated-caspase-3 polyclonal antibody (B) and DAPI (C) at 3 days after injury. Note that the cells stained positive for activated caspase-3 was found in the SG Z. (D and E) Fluorescence photomicrographs of the same animal section stained with antiactivated-caspase-9 polyclonal antibody (D) and DAPI (E) at 5 days after injury. The cells stained positive for activated caspase-9 exhibited nuclear fragmentation and were identified as a RGC by the FG staining (E, arrows). Scale bar = 25 μm. (DG) Inner nuclear layer (INL), inner nuclear band (GB), ganglion-cell layer.
Fig. 3. Caspase-3/7 inhibitors reduced nuclear fragmentation and caspase-3/7 activity. (a) Treatment with 1 μM Z-VAD-fmk or 1 μM Z-DEVD-fmk significantly reduced the number of cells with nuclear fragmentation at 3 h. This suggests that activation of caspase-3/7 played a role in the final demise of the dead cells. (b) Treatment with the caspase-3/7 inhibitor significantly reduced the number of cells with increased caspase-3/7 activity, **P < 0.01 vs. vehicle-treated group. *P < 0.01 vs. vehicle-treated group (mean ± S.E.M.).

The activation of caspase-3/7 following apoptosis.

Since caspase activation was delayed by several days following apoptosis, we examined if the Fas/DR5 pathway was implicated in the induction of caspase activation in axotomized rats. Activation of Akt downstream of Fas was examined in the brain of the BDNF- and Fas-deficient animals. We found no significant differences in Akt phosphorylation between the two groups. We also examined the role of Akt phosphorylation in the activation of caspase-3/7 in axotomized rats. We investigated the levels of Akt and the activity of Akt phospho-Akt signals at various time points after axotomy. Western blots analysis revealed that Akt levels remained unchanged up to 7 days post-axotomy (Fig. 4A). Phospho-Akt levels, on the other hand, increased as early as 3 h post-axotomy, peaked at 7 days, and remained elevated through 2 days (Fig. 4A and B). Akt phosphorylation returned to control levels at day 2, when caspase-3/7 activity returned to control levels (Fig. 4B).

When pAkt level was high, apoptosis proceeded when pAkt levels returned to control levels. To verify if the changes in pAkt level were attributable to changes in control group, these caspase-3/7 deficient mice were subjected to axotomy. The results were consistent with those obtained in the control group (Fig. 4C). This suggests that the axotomy-induced upregulation in Akt phosphorylation in normal animals was not observed in the control group.

Bromurocyclin or LY294002 injections did not induce caspase-3/7 activity.

To investigate the functional significance of the elevated pAkt levels following axotomy, intraventricular injections of 0.1 μM Wortmannin (WM) or 1 μM LY294002 (LY) were performed. Both WM and LY significantly increased phosphorylation of PI3K (Phan et al., 2000) and have been shown to lower pAkt levels in recent studies (Carmo et al., 2008). As expected, WM and LY injections significantly lowered pAkt levels in axotomized rats relative to that of the vehicle-injected group (Fig. 5A).

Quantification of the incidence of nuclear fragmentation in the GCL demonstrated that the fragmented nuclei observed in the axotomized group were not seen in the vehicle-injected group. WM and LY injection on the other hand, resulted in low levels of nuclear fragmentation in the GCL of unstimulated rats (Fig. 5B). Given the pivotal role of PI3K/Akt in neuronal survival, it is not surprising that reduction in Akt phosphorylation following injection of WM and LY resulted in slightly enhanced nuclear fragmentation in the absence of axotomy. While nuclear fragmentation was not detected in vehicle-injected rats at 2 days after axotomy (Fig. 5A), injection of vehicle led to the presence of a few apoptotic nuclei in one of the animals on day 2. The incidence of nuclear fragmentation was significantly increased in vehicle-injected rats compared to the vehicle-injected group. LY294002 injection also resulted in a significant increase in nuclear fragmentation, although this was not significant compared to the WM-treated group (Fig. 5B). This suggests that the inactivation of nuclear fragmentation between the WM- and LY-injected groups was possibly due to an unexpectedly high density of nuclear fragmentation in one of the WM-injected animals (density of 15.5 cells per mm² vs. the mean of 6.2 cells per mm² for the other three animals). Thus, the frequency of nuclear fragmentation induced by WM and LY in axotomized rats was significantly higher than those in normal rats (Fig. 5B). The marked increase in nuclear fragmentation in axotomized rats following axotomy suggests that Akt activation is crucial for the nuclear fragmentation of RGCs following axotomy. One may consider these data to indicate that partial inhibition of Akt activation was associated with a premature onset of apoptosis on day 2 post-axotomy, suggesting that PI3K activity is required for nuclear fragmentation after axotomy may serve as an early trigger for the progression of programmed cell death.

To examine if the WM or LY-induced apoptosis in axotomized rats exhibited caspase activation. WM- or LY-injected animals were killed at various time points and subjected to immunohistochemistry against caspase-3 and AP1. The staining of caspase-3-positive cells was not observed in the GCL, and was not observed in all layers of
Fig. 4. Phosphorylation of Akt following axotomy. (A) Western blot analysis of changes in phospho-Akt levels in neonatally retinas. An increase in the phosphorylated form of Akt at serine 473 can be detected as early as 1 day after axotomy. The levels of phospho-Akt peaked at 1 day and remained high through day 2. Akt levels remained unchanged following axotomy. The expression of β-actin was included to show that equivalent amounts of protein were present in each lane. Representative blots from three independent experiments performed in duplicate are shown. (B) A histogram representing the changes in phospho-Akt levels at axotomy time points after axotomy (N = normal, 1d = 1 day post-axotomy, 2d = 2 days post-axotomy). (C) and (D) Photomicrograph of the same retinal sections stained with DAPI (C) and phospho-Akt antibody (D) at 2 days. Note the phospho-Akt staining was observed mainly in the GCL. Scale bar = 25 μm. GCL = inner nuclear layer; INL = inner nuclear layer; OPL = plexiform cell layer.

Discussion

Activation of caspase-9 and normalized RGC death proceeded at a faster rate in hamsters.

Most of the earlier studies examining the apoptosis changes in neonatally RGCs have been carried out in rats. To facilitate comparison with previous studies, we characterized the temporal profile of normalized RGC death in hamsters. In this study, we found that normalized RGC loss in hamsters progressed at a faster rate compared to that in rats. Whereas only about 35% of the neonatally RGCs in hamsters were lost from 2 to 7 days in rats (Perentes-Karro et al., 1996), more than 65% were lost in hamsters. Although RGC loss was absent between day 3 and day 5 in hamsters, while b
occurs between day 3 and day 7 in vivo ( Schneider and Hall, 1999; Ponzetto et al., 1999; Wang et al., 2000; van der Geest et al., 1998). In addition, previous studies have shown that anoxia-induced RGC death does not contribute until day 5 in vivo (Ponzetto et al., 1999). Our study showed that RGC loss began at 3 days and progressed as evidenced by the initial appearance of fragmented nuclei at 3 days.

Concurrently, caspase-3 activation in the GCL was also observed on day 3. No caspase activation was observed at 1 and 2 days, indicating that caspase activation was delayed after axotomy damage. Our observations corroborate and extend findings from previous studies demonstrating activation of caspase-3 at 4-5 days after axotomy (Keirstead et al., 1999). By examining the temporal profile of caspase activation following axotomy, we verified that caspase activation was not observed before 3 days. In addition, we validated the labeled cells in the GCL to be RGCs by morphological labeling. However, we did not observe activation of caspase-3 in the retinal periphery, as was reported by Keirstead et al. (1999). This discrepancy could be due to improper differences, or differences in the sensitivity or specificity of antibodies used in the two studies. According to Ponzetto et al. (2000), the antibody used in Kerstett et al.'s study may also recognize activated caspase-7.

The high correlation of nuclear fragmentation and activated caspase-3 immunoreactivity observed in the current study suggests that these two caspases play a role in the morphological changes associated with apoptosis in anoxia-induced RGCs. This observation is in agreement with previous findings showing that caspase-3 cleaves substrates including PARP and Daxx, contributing to the morphological hallmark of apoptosis such as DNA fragmentation and chromatin condensation (Carmen et al., 1994; Martin et al., 1996; Tanaka et al., 1998). In addition, our data also suggest that the delayed activation of caspase-3 may contribute to the observed late onset of anoxia-induced RGC death. However, it should be noted that only about 50% of the fragmented nuclei remain positive for activated caspase-3 at all timepoints examined in the current study. In addition, caspase-9 inhibitors were more effective in lowering caspase-3 activation than inhibiting nuclear fragmentation. These observations suggest that other pathways may be recruited to eliminate anoxia-induced RGCs. Thus, inhibition of caspase-9, despite reducing caspase-3 activation by 50%, was only partially effective in preventing nuclear fragmentation. In agreement with this hypothesis, implication of caspase-3 in anoxia-induced RGC death is recently demonstrated (Witthaut et al., 2005). Finally, it is important to note that since caspase-9 shares substantial specificity with caspase-4 and -5 (Thornberry et al., 1997), the caspase-9 antibody used may also inhibit caspase-4 and -5. Therefore, the precise involvement of caspase acts from caspase-4 to -9 in the elimination of RGCs following axotomy.
at the time this and further experiments will be required for clarification.

Inactivation of the PI3K/Akt pathway in delaying the activation of caspase

Since the kinetics of caspase activation correlated with that of acutely

Neither apoptosis in vivo, providing evidence for the physiological

function of the PI3K/Akt pathway in the delayed activation of caspase

Seemann et al. (2004). It adhered to the phosphatidyl in response to

activate the PI3K/Akt pathway in vivo. In contrast, Akt activity

Alternatively, Akt was not activated to the same extent from


days post-
adhesion

[0]

Fig. 5. Schematic diagram illustrating the temporal sequence of Akt

activation and caspase activation in retinal explants. After 6 h

of axotomy, PI3K, but not the other PI3K isoforms, was inhibited.

encephalomyelitis (3-herpesvirus) (Tang et al., 2001). We have earlier

shown that inhibiting this increase possibly attenuated the kinetics

of apoptosis in vivo, providing evidence for the physiological

relevance of these results. The role of the caspase-3 and -9 activation

and/or expression of caspase-9 activation

In the current study, we investigated

the cell-autonomous role of PI3K/Akt in vivo, encephalomyelitis (3-herpesvirus)

and EAE have been shown to delay axotomy-induced RGC death by

activating the PI3K/Akt pathway (Kuroda et al., 2000b; Kuroda et al., 2000).

In this study, we found a rapid but transient increase in Akt

phosphorylation at 3 h post-injury. We subsequently showed that partial inhibition of this increase by W7 or LY294002

prevented some cells in the GCL to remain asymptomatic or in an earlier stage of apoptosis, by inactivating the PI3K/Akt pathway in the regulation of RGC apoptosis. Earlier activation of caspase-3 and -9 was demonstrated to take part in this premature apoptosis, indicating that the regulation of RGC death was achieved by limiting caspase activation. The drop in pAkt level on day 3 coincided with the onset of caspase activation and nuclear fragmentation at 3 days, suggesting that the lowered pAkt level may contribute to the continuation of the apoptotic cascade on day 3. These observations indicate that Akt phosphorylation may attenuate RGC death by limiting caspase activation in normal neural tissues. However, it should be noted that the use of W7 or LY294002 provides no information on the relative importance of PI3K and Akt activation in the inhibition of caspase. PI3K can potentially inhibit caspase activation by other signaling molecules. Nonetheless, the reduction in phospho-Akt levels by W7 and LY294002 injection suggests that Akt may act at least moderate some of PI3K's mitogen-activated protein kinase activity. The colocalization of Akt with the somata of RGC suggests that Akt is localized to the soma of RGC. Together, our data suggest that PI3K activity and/or the axon-sparing-induced increase in pAkt level may play a physiological role in attenuating RGC apoptosis by limiting activation of caspase-3 and -9.

A previous study examining Akt phosphorylation following axotomy showed that axotomy leads to a drop in pAkt level on day 4 in rats (Kuroda et al., 2000b). In the current study, we similarly observed a slight decrease in pAkt level following axotomy at 5 and 7 days, but the decrease was a lesser extent. By examining the time-points before RGC death, we maintained that axotomy-induced Akt phosphorylation preceded the lower pAkt content observed on days 3 and 7. Our data compare well with previous reports where axotomy-induced Akt phosphorylation in a rat model was shown to induce an increase in Akt activation (Munchow et al., 2001; Tang et al., 2001; Wang et al., 2000b). We have earlier shown that inhibiting this increase possibly attenuated the kinetics of apoptosis in vivo, providing evidence for the physiological relevance of these results. The role of the caspase-3 and -9 activation in axotomy-induced RGC death remained controversial. In the current study, treatment with W7 and LY294002 injections induced premature activation of both caspase-3 and -9. Since caspase-3 activation in axotomy-induced RGC occurred downstream of caspase-9 activity, the contribution to apoptosis by caspase-9 and -3 to caspase-3 induction likely indicates that the PI3K/Akt pathway possibly inhibited caspase activity in vivo. In conclusion, Akt overexpression showed the same inhibitory effect on both inactive and active forms of human caspase-9 via phosphorylation at Serine 196 (Kuroda et al., 1999). Although a previous report showed that the Akt phosphorylation site on human caspase-9 is shown in vitro and in vivo (Kuroda et al., 1999), a recent study showed that Akt can block caspase-3 and -9 activation in the presence of unphosphorylated caspase-9 in a cell line expressing rat caspase-9 (Tanaka et al., 2001). It is possible that PI3K may directly affect the activation of caspase-9 via other yet unidentified mechanisms. Furthermore, whether the Akt phosphorylation site is present in the human caspase-9 remains to be elucidated. Finally, it cannot be ruled out that the PI3K/Akt
pathway may also exhibit direct inhibitory effect on caspase-3, independent of caspase-9 activity. Delineating these possibilities will expand our understanding on the regulation of caspase activity in autoreactive retina and thus increase our ability to design effective treatments for degenerating RGCs in lesions like glaucoma.

In conclusion, findings from this study demonstrated that caspase-3 and -9 activation and nuclear fragmentation occurred with a delay of 3 days following optic neuritis, coinciding with the beginning of RGC death post-neuropathy. Elevated levels of phospho-Akt were observed as early as 3-4 days post-neuropathy. Partial inhibition of the FUR/Akt pathway contributed to premature onset of nuclear fragmentation, accompanied by the early activation of caspase-3/9 in the RGC. These observations suggest that the activation of the FUR/Akt pathway may be implicated in preventing the initiation of caspase-3 and -9 and delaying the onset of caspase-induced apoptosis to 3 dpa (Fig. 4c). Our data suggest that the intraperitoneal injection of Akt activation may play a physiological role in preventing the activation of caspases in vivo. Further study of the period of delayed Akt phosphorylation, for example, may serve as a feasible treatment for prolonging autoreactive RGCs or in diseases such as glaucoma.

Experimental methods

Optic nerve transection

A total of 142 adult male Long-Evans rats (Taconic Farms, Inc.) were used in this study. All operations were performed on animals anesthetized with intraperitoneal injection of sodium pentobarbital (Dial奉, 150 mg/kg). Unilateral optic nerve transection at 1.5 mm from the optic disc was performed as previously described (Bouchard et al., 2001). Surviving RGCs were retrogradely labeled by placing a piece of gelatin (Upjohn, Kalamazoo, MI) soaked with 1% fluoroquinal (Fluoromax, Denver, CO) at the outer surface immediately after the operation. Animals with compromised blood supply after the operation, as determined by examining the fundus under an operating microscope, were excluded. Operative animals were housed with an inverter of artificial illumination, followed by transcardial perfusion with 4% paraformaldehyde (PFA).

Quantiﬁcation of surviving RGCs at various time points following surgery

To examine the density profile of surviving RGCs, the left eye of the unilaterally transected animals were enucleated at 1, 2, 3, 4, 7, 10, and 14 days post-neuropathy (dpa; n = 4–6 per group). The retinas were dissected in 4% PFA and prepared as flatmounts as previously described (Bouchard et al., 2001). Flat-mounted retinas were examined under fluorescence microscopy using an ultraviolet filter (excitation wavelength = 330–380 nm). Labelled RGCs were counted along the median line of the fourth quadrant starting from the optic disc to the peripheral border of the retina at 90-μm intervals, under a magnification of 200 x. The mean density of labeled RGCs in each retina was then multiplied by the area of the retina to obtain the number of labeled RGCs in the retina.

Immunohistochemistry

The left eyes of all animals prepared for immunohistochemical analysis were enucleated following transcardial perfusion with 4% PFA and subsequently post-fixed in the same fixative for 4 h. Transcardial injections of 1% paraformaldehyde were made via the right ventricle following the method described by Jentsch et al. (1993). The sections were cut transversely along the superior-inferior axis of the eyeball and stored at −20°C until use. Only sections containing the optic nerve stump were used in this study to ensure comparability.

For immunohistochemical detection of activated caspase-3 and activated caspase-9, animals were divided into three subgroups: (1) unoperated animals to serve as control (n = 4); (2) animals that have received optic nerve transection and FG-labeling of RGCs, sacrificed at 1, 2, 3, 5, 7, 10, and 14 dpa (n = 6–8 per group); (3) animals that have received surgery without FG-labeling, sacrificed at 1, 2, 3, and 5 dpa (n = 4). Animals in group 3 served as negative controls in verification of the immunoreactivity staining from FG interference.

Retinal sections were thawed, air-dried, and washed three times in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The sections were first blocked with 3% normal goat serum (NGS) in 0.3% Triton X-100 at room temperature for 20 min. The slides were then incubated with anti-phospho- doubly reactive antibody (1:50) and anti-activated caspase-9 polyclonal antibody (1:100) Cell Signaling Technology, Beverly, MA) or anti-activated caspase-9 polyclonal antibody (1:100 Cell Signaling Technology) in NGS 0.3% Triton X-100 at room temperature for 4 h. After washing, sections were washed and activated caspase-9 staining was visualized by Texas Red-conjugated goat anti-rabbit secondary antibody at 1:50 (Vector Laboratories, Burlingame, CA). All slides were counterstained with 1.6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO) in nuclear neuronal morphology. Paranuclear slides were covered with Vectashield ® mounting medium for fluorescence (Vector Laboratories) and examined under microscope.

Quantiﬁcation of labeled RGCs, activated caspase-3 and -9 immunoreactivity in sections

The number of FG-labeled RGCs, immunoreactive cells (for activated caspase-3 and -9) and fragmented nuclei in each retina was counted. The number of cells exhibiting co-localization of any two of the mentioned parameters was also counted. Counting was done on three retinal sections per animal to increase the reliability of the data. The numbers counted from the three sections were added up to give the final number of labeled cells in each retina. Since all the retinal sections were used in this study combined the optic nerve stump, the length of all the retinal sections was comparable (8000 ± 200 mm). The number of labeled cells in each retinal section was therefore used as an estimation of the density of labeled cell. FG-labeled RGCs and DAPI-stained nuclei were examined using an ultraviolet filter (excitation wavelength = 450–490 nm). The color of the two dye signals for FG and blue-violet for DAPI were readily distinguished from each other under the same filter (Fig. 2B). Fragmented nuclei were identified as described by earlier reports (Bouchard et al., 2001). Immunoreactive cells were identified as small, roundly

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and fragmented nuclear structures that are brightly labeled by DAPI. Nuclear fragmentation and formation of apoptotic bodies are morphologically associated with a loss of adhesion of RGCs. Untreated animals were subcutaneously injected with 0.2 l of PBS (pH 7.4), and wounded rats were treated with 0.5 ml of PBS (pH 7.4) or 5 ml of 0.5% HEPES-KOH (4 mg/ml, immediately after surgery) at day 8 and on day 2 after injection. Animals were sacrificed at 3 days and the operated eyes were removed and processed for immunohistochemical analysis against activated caspase-3 followed by DAPI staining. The number of cells exhibiting nuclear fragmentation and activated caspase-3 immunoreactivity in each group was counted.

To examine the effect of MEK inhibition on nuclear fragmentation and apoptosis, cultured neuronal cells were used. Cells were plated at 1.5 x 10^5 cells per plate on 12 wells in DMEM media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were then treated with 100 nM PD98059 or 100 nM SP600125 for 3 days. The percentage of cells with fragmented nuclei and activated caspase-3 was measured using 4,6-diamidino-2-phenylindole (DAPI). The number of nuclear fragments was counted using a fluorescent microscope.

Western blot analysis

Procedures for Western blot analysis were adapted from the manufacturer's protocol. Briefly, a thymine kinase positive vector (pepA) containing the thymidine kinase (TK) gene was used to transfect SK-BR-3 cells. The transfected cells were then treated with 1000 nM of 6-mercaptopurine (6-MP) for 48 hours. The cells were then harvested and lysed in lysis buffer containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% Nonidet P-40, and 50 mM sodium phosphate buffer, pH 7.4. The lysates were then subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in PBS and probed with anti-6-MP antibody (1:1000) followed by HRP-conjugated secondary antibody (1:5000). The blots were developed using ECL chemiluminescence detection system. The chemiluminescence images were captured using a digital camera and quantified using ImageJ software. The data were analyzed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. Differences were considered significant at p < 0.05.

Data are given as means ± standard error of mean (SEM). Statistical significance was evaluated by Student's t-test for comparisons between two groups or by one-way ANOVA, followed by Tukey-Kramer post hoc test for comparisons among three or more groups. Differences were considered significant for p < 0.05.
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Role of Nitric Oxide in Pain Fan Sai, Pui, Ip Huy, Lee, Ansley W. Medical School, The Chinese University of Hong Kong, Hong Kong The evidence for the role of nitric oxide (NO) in mediating pain signals has been conflicting. Earlier studies using monocyte nitric oxide synthase inhibitors (NOS) indicated that NOS plays an important role in modulating pain. However, more recent studies have shown dissociation between NOS inhibition and pain behavior. In the present study, we have investigated NOS activity in peripheral nerve and spinal cord by selective inhibition of NOS. In a rat model of mechanical allodynia, exogenous NO synthase inhibitor L-NAME was administered. Following administration of NOS inhibitor, mechanical allodynia in rats was induced by light touch of the left ulnar and L5 spinal nerves. The degree of mechanical allodynia was measured by the paw withdrawal test for pain flexing with von Frey Monofilament. NOS activity determinations and western blot analysis showed that normal form of NOS was the major isoform (iNOS) in spinal cord from normal and acupunacupunctured animals. Treatment with L-NAME induced L-NAME, piperazine and L-NAME, piperazine + L-NAME produced a significant increase in response pain threshold. Since determination of NOS activity and inhibition of pain response by non-invasive and highly specific NOS selective pharmacological inhibitors suggest that NOS is additively involved in nociceptive pain.

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Effect Of Electro-Acupuncture On Nitric Oxide Synthase Activity In The Brain Of The Rat After Stroke Lai W P Leung, Fook K W Su*, Poem Yew, Samuel L Le Department of Neurological Sciences, The Hong Kong Polytechnic University, Hong Kong Department of Applied Science, The Hong Kong Polytechnic University, Hong Kong Department of Applied Science, The Hong Kong Polytechnic University, Hong Kong Department of Applied Science, The Hong Kong Polytechnic University, Hong Kong

This study used a sample study in order to investigate the effect of electro-acupuncture (EA) on the functional activity of Nitric Oxide Synthase (NOS) using the substrates assay method. The expression of different isoforms of NOS was investigated by the Western blotting method. Brain samples NOS and synaptoneurospasm were collected from rats with stroke and was the body weight of 250-450g. The left cerebral ventricles and the MCAs of cerebri were exposed and completely excised for 1h, then reperfused for 22 hours. The rats were then killed. Each rat was subjected to different treatments, CB10 or P60 for 30 minutes immediately after the end of MCA occlusion. No EA was applied to the control group. The left hippocampi were used for analysis. The mean functional activity of NOS (spinal nitric oxide) was significantly increased after local EA treatment and recovery (p<0.05) when compared to the non-acupuncture group. The activity of NOS was significantly decreased when EA was applied to CB10 (p<0.05) or significantly increased when EA was applied to P60 (p<0.05) for the non-stroked and synaptoneurospasm conditions. However, there was no significant difference in NOS activity when EA was applied to CB10 and significantly increased NOS activity when EA was applied to P60. These results show that EA may modulate the activity and the expression of NOS under different conditions and may modulate its function through different mechanisms.
was added with 1, 3, 5-tribromo-1, 3, 5-trifluorocyclohexane to attach a bifunctional group at the 3-position of the NMR spectra of 5-iodoquinolinone. The resulting 3, 5-difluoroquinolinone exhibited a broad range of reactivity and stability under a variety of conditions.

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Enhanced Electronic Flux and Reduced Collinoidal Disaccharide Metabolism May Explain Calcium-Independent cAMP Activation by Fluorophorosine


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Calcium-dependent nitric oxide synthase (NOS) is glucose-galactose, cyclic adenosine 5'-monophosphate (cAMP) is an enhancer of NO production constitutively, in the absence of an intact NOS. Here, we examine the potential role of NO in the regulation of cAMP levels. NOS inhibitors, including 7-nitro-2-aminobutyric acid (NBA) and 7-nitro-benzo-2-aminobutyric acid (NBA), reduced cAMP levels to a similar extent. In addition, the addition of NBA to the reaction mixture resulted in a decrease in cAMP levels. On the other hand, the addition of NBA to the reaction mixture resulted in an increase in cAMP levels. These results suggest that the inhibitory effect of NBA on cAMP production is reversed by increasing the concentration of NBA in the reaction mixture. The results suggest that the inhibitory effect of NBA on cAMP production is reversed by increasing the concentration of NBA in the reaction mixture. The inhibition of cAMP production by NBA is not reversed by increasing the concentration of NBA in the reaction mixture. The inhibition of cAMP production by NBA is not reversed by increasing the concentration of NBA in the reaction mixture. The inhibition of cAMP production by NBA is not reversed by increasing the concentration of NBA in the reaction mixture. The inhibition of cAMP production by NBA is not reversed by increasing the concentration of NBA in the reaction mixture. The inhibition of cAMP production by NBA is not reversed by increasing the concentration of NBA in the reaction mixture. The inhibition of cAMP production by NBA is not reversed by increasing the concentration of NBA in the reaction mixture. The inhibition of cAMP production by NBA is not reversed by increasing the concentration of NBA in the reaction mixture.
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Inhibition of Mitochondrial Respiration By Nitric Oxide Also Decreases Synthesis Of UTP And CTP

Chade Blank, Randa Kheir, Catherine Lemaire, Melkote, John; Virginia Commonwealth University (VCU) School of Medicine, Richmond, VA

Nitric oxide inhibits mitochondrial function in a variety of cell types. We studied the effects of NO on mitochondrial respiration and adenosine triphosphate (ATP) formation in isolated rat liver mitochondria. NO was generated by the NO-synthase activity of nitric oxide synthase (NOS) in a tissue culture plate. Mitochondria were isolated from the livers of rats treated with NOS inhibitors or NOS agonists. The mitochondrial NOS activity was measured by monitoring the formation of nitrite in the culture medium.

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L-Ascorbic Acid Potentiates Endothelial Nitric Oxide Synthesis By Increasing Intracellular Thioredoxin Reductase Levels

Regina Hever, Amya Uthman, David E. Weir, James M. Turrentine, Arizona State University, Tempe, AZ

Endothelial nitric oxide synthase (eNOS) is a key enzyme in the regulation of blood flow and blood pressure. Recent studies have shown that L-ascorbic acid (LA), a natural antioxidant, can potentiate eNOS activity. In this study, we investigated the effects of LA on eNOS activity and the underlying mechanisms in cultured endothelial cells.

LA treatment significantly increased the intracellular levels of thioredoxin (Trx), a small protein that functions as a redox regulator. Trx is known to play a role in cellular redox balanced and in eNOS activation. We found that LA treatment increased the Trx levels and that this effect was associated with an increase in eNOS activity. These findings suggest that LA might be a promising therapeutic agent for the treatment of endothelial dysfunction.

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Perfluorinated Hemoglobin Polymers (PHP): A Novel Oxyhemoglobin-Generating Agent For The Treatment Of Nitric Oxide-Induced Strokes


Perfluorinated hemoglobin polymers (PHP) are a novel class of oxygen delivery agents that are currently under investigation for the treatment of nitric oxide-induced strokes. These agents have been shown to increase local oxygen delivery to ischemic tissues, potentially improving neurological outcomes.

In this study, we investigated the effects of PHP on oxygen delivery and tissue metabolism in a mouse model of nitric oxide-induced stroke. Our results showed that PHP treatment significantly improved oxygen delivery and reduced tissue damage, indicating the potential therapeutic value of this novel oxygen delivery agent.
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Methods: Fourteen white rabbits receiving intravenous injection of 0.7 ml isotonic noradrenaline blood at 0.7 ml/s were divided into two groups as follows: (1) saline injected (control group, n = 7); (2) blood injected (SAH group, n = 7). The rabbits were sacrificed after 72 h. and the hippocampal region, brain stem and cerebellum were examined. The brains were prepared for the spectrophotometric determination of superoxide dismutase (SOD), catalase (CAT) activities and thiobarbituric acid reactive substances (TBARS) levels.

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U/g)</th>
<th>CAT (U/g)</th>
<th>TBARS (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C group</td>
<td>45</td>
<td>150</td>
<td>0.18</td>
</tr>
<tr>
<td>SAH group</td>
<td>66</td>
<td>21</td>
<td>0.45</td>
</tr>
</tbody>
</table>

**SOD** and **CAT** activities and **TBARS** levels were found significantly higher in SAH group compared to the control group.

Conclusion: These data provide evidence of a marked lipid peroxidation and enhancement of brain antioxidant activity in brain tissue suggesting SAH-induced free radical production which may be partly responsible for neuronal and subsequent ischemia in SAH.

EFFECT OF ELECTRO-ACUPUNCTURE ON ATTENUATING EXPERIMENTALLY INDUCED CEREBRAL ISCHEMIA-REPERFUSION MODEL IN RATS

Elsa Kae-Wu Shi; Samuel Chun-Lap Lo; Mason Chin-Pang Leung

Background and Purpose: Electro-acupuncture (EA) has become increasingly popular as a therapy for stroke rehabilitation. However, its effect has only been evaluated at the acute stage (within 24 hours) and not at the subacute stage (1-7 days). The purpose of this study is to investigate the efficacy of EA on the ischemic state at the subacute stage of cerebral ischemia and to document the significance of time by intervention. Methods: Spine-vehicle rats were randomly assigned into three groups: Normal group (n=17), Ischemia group (1-hour right MCAO, EA-GRO group (30-minute EA at 500Hz, GR20, immediately after MCAO). Rats were killed at 1 to 7 days (8 per time-point) after the onset of MCAO. Brains were harvested for measuring the total activity of nitric oxide synthase (NOS), amount of malondialdehyde (MDA) and expression of transforming growth factor (TGF-B1). Results: Total NOS activity was suppressed in the EA group at day 1 onwards (day 1 to day 5, p<0.05) and day 3 to day 7, p<0.05 when compared to ischemia group. But the MDA amount was not found.

SFR 2002-Paris S123
LYMPHOCYTE ACONITASE ACTIVITY IS REDUCED IN ALZHEIMER DISEASE AND Mild COGNITIVE IMPAIRMENT


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Background: A body of experimental evidence suggests that in Alzheimer disease (AD) pathogenesis an important role is played by oxidative stress, but there is still a paucity of data on in vivo markers of free radical-induced damage. Aconitase is a Fe-S protein contained in mitochondria and in cytoplasm that catalyzes the interconversion of citrate and isocitrate via cis-aconitate. This enzyme is particularly sensitive to free radicals, such as superoxide anions and nitric oxide, and it could be inactivated in a condition of oxidative stress. Objective: To evaluate and compare aconitase activity in lymphocytes from AD patients, subjects with mild cognitive impairment (MCI) and healthy controls. Subjects: Thirty AD patients, sixteen MCI subjects, fourteen healthy old and thirteen young controls were studied. Methods: After extraction, lymphocytes were isolated and aconitase activity was measured spectrophotometrically at 240 nm. Activity is expressed as nM Fe$^{2+}$/minute. Results: AD showed the lowest aconitase activity compared to MCI (p<0.01), old (p<0.0001) and young (p<0.0001) controls. Also MCI significantly differ from both control groups (p<0.0001), while there was no difference between elderly and young healthy subjects. No relationship was found between age and aconitase activity in each group as well as in the pooled control group. Females always showed a significantly higher activity when compared to males of the same group. Conclusions: These preliminary results support the hypothesis that 1) markers of oxidative stress can be detected peripherally; 2) MCI is a condition at risk for dementia also from a biological point of view; 3) females are more resistant to oxidative stress.
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**ABSTRACT**

The antioxidant effects of electro-acupuncture (EA) on the oxidative stress in adult rats after cerebral ischemia were investigated. The results indicated that EA significantly reduced the levels of lipid peroxidation and the activity of antioxidant enzymes. These findings suggest that EA may be a useful method for preventing oxidative stress in the brain after cerebral ischemia.

**Keywords**: Electro-acupuncture, Cerebral ischemia, Oxidative stress.
ABSTRACTS

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Nail acid synthases (NOS) are a class of enzymes that catalyze the conversion of L-arginine to L-citrulline and NO. The NO produced by NOS is thought to modulate a variety of physiological functions, including blood pressure, neurotransmission, and inflammation. However, the regulation of NOS activity and its role in physiological processes are still not fully understood. In this study, we investigated the effects of treatment with L-arginine on the expression of NOS in rat heart tissue. We found that treatment with L-arginine significantly increased the expression of NOS in heart tissue, suggesting that NOS activity is upregulated by L-arginine. These findings provide new insights into the mechanisms of NO production and its role in cardiovascular health.

The Influence of Substrate and Nerve Density on the Inhibition of Endothelial and Neuronal Nitric Oxide
Synthase

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Nitric oxide synthase (NOS) plays a critical role in the regulation of blood pressure and vascular tone. The release of NO from NOS is dependent on the availability of L-arginine, the substrate of NOS. We investigated the effects of substrate availability on the inhibition of endothelial and neuronal nitric oxide synthase (eNOS and nNOS) in rat aorta and hippocampus, respectively. Our results showed that L-arginine deprivation significantly decreased the activity of eNOS and nNOS. These findings suggest that substrate availability is a critical factor in the regulation of NOS activity.
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ABSTRACTS

It is known that a recent and effective treatment should be identified in order to shorten the recovery period in Middle cerebral artery ischemia. Nitric oxide synthase (NOS) and transforming growth factor-beta 1 (TGF-β1) are two important markers of the delayed brain damages. The results demonstrated that the post ischemic administration of 1500PPM nitroglycerine in rats significantly improved the recovery period of the transient cerebral ischemia model. The effect of NOS inhibition on the neural recovery was assessed by the behavior in the delayed memory test. The NOS activity was significantly increased by the administration of nitroglycerine in the ischemic brain. The results suggested that the post ischemic administration of nitroglycerine could reverse the delayed memory impairment.

An effective treatment could be identified in order to shorten the recovery period in Middle cerebral artery ischemia. Nitric oxide synthase (NOS) and transforming growth factor-beta 1 (TGF-β1) are two important markers of the delayed brain damages. The results demonstrated that the post ischemic administration of 1500PPM nitroglycerine in rats significantly improved the recovery period of the transient cerebral ischemia model. The effect of NOS inhibition on the neural recovery was assessed by the behavior in the delayed memory test. The NOS activity was significantly increased by the administration of nitroglycerine in the ischemic brain. The results suggested that the post ischemic administration of nitroglycerine could reverse the delayed memory impairment.

New insights into the mechanism of the second step of NO formation revealed by the structures of nNOS and mNOS complexes with NADPH and NADH dehydrogenases.

Hajime U, Hitomi H, Mark P, Mihoko H, Jun-ichi S, Masao H, Tetsu H, Tadashi K, Show Yoshino, Toshio L, Yoko M, Takeshi T, Department of Molecular Biology, Kobe University Graduate School of Medicine, Kobe, Japan.

Nitrile oxide synthase (NOS) is a class of enzymes found in microorganisms, plants, animals, and humans. Inhibitors of NOS have been used to treat a variety of diseases, including heart disease and cancer. However, the exact mechanism of NO formation is not fully understood.

The authors investigated the mechanism of NO formation by combining X-ray crystallography and computational chemistry. They found that NOS complexes with NADPH and NADH dehydrogenases exhibit a novel mechanism for NO formation.

The results suggest that NOS complexes with NADPH and NADH dehydrogenases exhibit a novel mechanism for NO formation. This mechanism is different from the previously proposed mechanism, which involves the reduction of NO by a dihydrodiol of the heme moiety.
PREVENTIVE EFFECT OF ELECTRO-ACUPUNCTURE ON CEREBRAL ISCHEMIA-REPERFUSION INDUCED IN RATS

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Introduction: Electro-acupuncture (EA) has become increasingly popular as a therapy for stroke rehabilitation. Also it is always suggested to be beneficial to health for a preventive purpose. However, the role of EA as a preventive measure to stroke remains unclear. The aim of this study is to investigate the effects of pre-ischemic EA on the oxidative stress of cerebral ischemia and to document the significance of acupoints selection.

Materials: Sprague-Dawley rats were randomly assigned into four groups: Placebo (rats received anesthesia instead of EA; n=5), Non-acupoint (rats received EA on non-acupoints; n=5), EA-GB20 (rats received EA on Feng-chi, GB20; n=5) and EA-ST36 (rats received EA on Zu-san-li, ST36; n=5). Each group was subjected to nine sessions of 30-min treatment before 1-hour middle cerebral artery occlusion (MCAO). All the rats were killed at day 4 from MCAO. Brains were harvested for assaying the total activity of nitric oxide synthase (NOS), amount of malonaldehyde (MDA) and expression of transforming growth factor beta-1 (TGFβ-1).

Results: There was no significant difference of total NOS activity between the Placebo group and Non-acupoint group and the EA-GB20 group. Only the EA-ST36 group demonstrated a significant increase in total NOS activity (p<0.05). But the amount of MDA was not found to be significantly differed among all tested groups. However, electro-blotting revealed that TGFβ-1 was highly expressed in EA-ST36 group, but not in other groups (p<0.001).

Conclusions: Our result suggests that pre-ischemic electro-acupuncture at ST36, not GB20, is beneficial to cerebral ischemia.
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ELECTRO-ACUPUNCTURE COULD REGULATE THE EXPRESSION AND TOTAL ACTIVITY OF NITRIC OXIDE SYNTHASES INDUCED IN CEREBRAL ISCHEMIA

Ka-Wai Siu*, Samuel C.L. Lo† and Mason C.P. Leung*
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Background and purpose: Electro-acupuncture (EA) has been identified as an alternative treatment to stroke patients in China. However, the time to deliver EA may vary from several hours to days from the onset of ischemia. The present study is therefore to investigate the significance of time on exerting the beneficial effect of EA on ischemic rats. Methods: Sprague-Dawley rats were randomly assigned into six groups: Normal (rats received no treatment; n=17), Ischemia (Rats with 1-hour MCAO only; n=8), 9-day delay ischemic rats treated with immediate EA; n=5), 1-day delay (ischemic rats treated with EA at post-injury day 1; n=5); 2-day delay (EA at posts ischemia day 2; n=5); and 3-day delay (EA at post-ischemia day 3). EA at GB20 (Fengchi) was applied for 30 minutes at 2 Hz. All rats were killed at day 4 from MCAO. Brains were harvested for assaying the total activity of nitric oxide synthase (NOS) and the expression of three isoforms (iNOS, eNOS and nNOS).

Results: There was no significant difference of total NOS activity between the Normal group and all the delay groups (p>0.05). The total NOS activity was detected at similar level among all the delay groups. However, significantly reduced activity was observed on the delay groups in comparing to the ischemic group (p<0.05). Reduced expression of NOS isoforms was also observed after applying EA, which was not governed by the time of treatment.

Conclusion: Our study found that the electro-acupuncture could reduce NOS activity by suppressing their expressions. This beneficial effect was not affected by the time of intervention, which implied more flexibility of EA on clinical practice.
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Assessment of the cyc1 defect by lasertrapping

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Introduction: The cyc1 defect is the cause of the mutation in the cdc2 gene. Therefore, the defect in the cdc2 gene is a possible cause of the cyc1 defect. However, the mechanism of the cyc1 defect is not known. This study was to investigate the mechanism of the cyc1 defect by lasertrapping.

Materials and methods: The mechanism of the cyc1 defect is not known. This study was to investigate the mechanism of the cyc1 defect by lasertrapping.

Results: We analyzed the distance between the laser trap and the cell membrane using the distance between the laser trap and the cell membrane. We observed that the distance between the laser trap and the cell membrane is not significantly different between the laser trap and the cell membrane.

Discussion: We observed that the distance between the laser trap and the cell membrane is not significantly different between the laser trap and the cell membrane. This result suggests that the mechanism of the cyc1 defect is not known. Further study is required to investigate the mechanism of the cyc1 defect.
induced cell death. PND 7 intact male and female hamster pups were generated in N-Methyl-IKken and, the right facial nerve was transected at the eye, the experiment 1 consistent with the findings of other studies. Separate males were either implanted with a subcutaneous, non-implantable IP pellet, a placebo pellet, or not implanted. All 6-week-old rats were exposed to 30°C ambient temperature. The results demonstrated that IP administration is effective in animal studies, with a significant decrease in mortality. It is concluded that this technique is effective in reducing experimental harm from exposure to toxic substances.

7.4.5

Computer Analysis of Apoptosis in 7-NSSL Cells Under Cancer Therapies: Targeting Photochemistry of Photochemical Therapy

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These data are presented to study dynamic molecular interactions in a two-dimensional molecular model. The model is limited to a range between 30°C and 40°C, during treatment. The model results are compared with a two-dimensional mathematical model (R2M) and with the same mathematical model (R2M). The hypothesis is that the two-dimensional model is superior to the current understanding of the model. Patterns can be taken in those three-dimensional, between filter and buffer, and in a two-dimensional filter. The full length of the filters is limited by a given band of enzyme. Examples of filters showing the differences in color and 100% survival will be presented. The Open LBD software program has the capability of an interactive analysis of the data and is a function that we are currently developing. Supported by NIMH MH 55160.

7.4.6

Effects of aluminum on programmed cell death (apoptosis) and lymphocyte proliferation. Relevance for Alzheimer's disease

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Age-related environmental risk factors for Alzheimer's disease (AD) (Aluminum): aluminum may have been extensively investigated as a neurotoxic substance. Epidemiological studies have been done to determine the age-related risk for Alzheimer's disease. In a study of 111 patients with AD, aluminum was found to be a neurotoxic substance. The results of this study are consistent with the hypothesis that aluminum may be a risk factor for Alzheimer's disease.
Effect of electroacupuncture on reducing lipid peroxidation by increasing activities of antioxidant enzymes after transient focal cerebral ischemia

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This study is to investigate the effect of electro-acupuncture (EA) on regulating the activities superoxide dismutase (SOD), glutathione peroxidase (GPx) and lipid peroxidation (LPX) after transient focal cerebral ischemia. 36 rats were divided into 5 groups: Normal (n=8), Normal-EA (normal rats with EA, n=4), Ischemia (1hr MCAO, n=8), Ischemia-Sham EA (Ischemic rats with Sham EA, n=8), and Ischemia-EA (Ischemic rats with EA, n=8). EA was applied on Fengchi for 30 min at 2 Hz. Brains were harvested at post-ischemia day 1. Total SOD and GPx activities were not altered between Normal and Normal-EA group. Induction of ischemia could increase SOD and GPx activities in other three groups (P<0.05), but not reduce LPX level. Compared to Ischemia group, Ischemia-EA group exhibited up-regulated SOD and GPx activities by 30% and 10%, respectively, resulting in lowered LPX level (P<0.05). It implied that EA could potentiate enzyme activities that ameliorated LPX level. EA-induced effect was not observed in Ischemia-Sham EA group, suggesting that electrical stimulation was essential to produce the neuroprotective effect. This study concluded that EA could provide an appropriate balance of SOD and GPx activities to attenuate LPX after cerebral ischemia in rats. This research was supported by the Faculty Area of Strategic Development Grant (A106) and the Central Research Grant (C-W016) from Hong Kong Polytechnic University.
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