The Hong Kong Polytechnic University

Jockey Club Rehabilitation Engineering Centre

Evaluation of post-stroke rehabilitation using neuromuscular electrical stimulation and gait training in a rat model of focal cerebral ischemia

LEUNG Lai Yee

A thesis submitted in partial
fulfilment of the requirements for
the Degree of Master of
Philosophy

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CERTIFICATE OF ORIGINALITY

I hereby declare that 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 that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

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March 2006

DECLARATION

I hereby declare that all research designs, analysis and written works (including interim reports, conference papers, peer-reviewed paper and thesis), experimental set-up and procedures described in this thesis, except part of the microdialysis experiments*, were done by me.

*The microdialysis experiment was designed by me and approximately 20% of works on microdialysis were done with the helps from the colleagues at Zhejiang University – Mr SM Zhang, Mr XH Zeng and Ms KP Zhang. Their helps are very much appreciated.

LEUNG Lai Yee

March 2006

ABSTRACT

Treadmill exercise and neuromuscular electrical stimulation (NMES) were commonly used as strategies in stroke rehabilitation. Clinical studies have revealed the functional and physiological benefits of treadmill exercise in people after stroke. Recent animal studies showed that early rehabilitation (24hours after stroke) reduced brain damage in rat after stroke, but some studies showed exacerbation of infarction. The beneficial effects of early intervention of exercise on stroke are still controversial. Many studies have demonstrated that NMES improved muscle strength and gait. Functional brain imaging studies showed activation of cerebral cortex when NMES was applied in healthy subjects. The *in vivo* effects of NMES has not been well studied. The objective of this research was to investigate the neurological, functional and physiological effects of treadmill training and NMES in rehabilitation of ischemic stroke using a rat model.

In this study, focal cerebral ischemia was induced by occluding middle cerebral artery using intraluminal suture technique in rats. A total of 55 rats were included in the study and they were assigned into sham-operated group (Sham), control group (Control), Exercise group (EX) and electrical stimulation group (ES). Two-week treadmill exercise and NMES were prescribed 24 hours after

stroke to EX group and ES group respectively. Sham (without stroke) and Control groups (with stroke, but no intervention) were remained in cages for two weeks. Body weight was measured daily as an indicator of general physiological status of the rats after stroke. Postural reflexes and limb placing tests, which were used to detect the improvement in neurological deficits caused by stroke, were performed daily. Brain weight, infarction volume, brain edema index were measured to evaluate the brain damage or recovery after interventions. Wet weight of affected tibialis anterior was used to indicate muscle atrophy or growth. In the microdialysis study, cerebral spinal fluid from right hippocampus of the rats was collected *in vivo* before, during and after interventions on day 1, 2, 4, 7 and 14 after stroke. Concentration of aspartate, glutamate, taurine and gamma-aminobutyric acid (GABA) were obtained by high performance liquid chromatography (HPLC) with fluorescence detection. These are common excitatory (aspartate and glutamate) and inhibitory (taurine and GABA) neurotransmitters in mammalian central nervous system and they involved in many mechanisms such as cerebral ischemia and physical activities.

Firstly, the results showed that treadmill exercise might facilitate functional recovery. Secondly, NMES might reduce brain damage and atrophy on ipsilesional hemisphere of the brain. It also showed significant increase in the

wet weight of the stimulated muscles. Thirdly, there were increases in hippocampal glutamate and aspartate levels during interventions in Ex group, as shown by other studies. However the increases did not persist with days. Only small increases in these two neurotransmitters were observed during the first week in EX and ES groups. The increased levels were lower than the excitotoxic levels, which might not be enough to exacerbate brain damage after ischemic insults. Finally, the levels of taurine and GABA were suppressed by both interventions.

In conclusion, treadmill exercise facilitated functional recovery, whereas NMES increased muscle mass on the affected side and reduce brain damage and atrophy after stroke. Moreover, natural recovery in Control group might be triggered by ischemic injury. This ischemia-induced recovery might be dominant during the 1st week after ischemic insults in all groups. In addition, temporary and small increase in glutamate and aspartate might not worsen the brain damage. Finally, taurine might play an important role in natural recovery, but not in recovery triggered by the interventions. The findings of this study contribute to further investigations on effects of different strategies of rehabilitation on ischemic injured brain and also provide scientific basis for the underlying mechanisms of motor recovery after ischemic stroke.

Publications arising from the thesis

1. LY Leung, SM Zhang, XH Zeng, KP Zhang, XX Zheng, KY Tong. Neurochemical effects of exercise and neuromuscular electrical stimulation on brain after stroke: a microdialysis study using rat model. *Neuroscience Letters*. 2006 Apr 10;397(1-2):135-9.

2. LY Leung, KY Tong, XX Zheng, AFT Mak. Effect of treadmill exercise on physiological outcomes after cerebral ischemia in rats – A pilot study. The 2nd World Congress for Chinese Biomedical Engineers. Beijing. China. Sept 2004.

3. Leung LY, Zhang SM, Zeng XH, Zhang KP, Tong KY. Neurochemical effect of neuromuscular electrical stimulation in brain after stroke: A microdialysis study using rats with focal cerebral ischemia – A pilot study. 10th Annual Conference of the International FES Society. Montreal. Canada. July 2005.

4. XL Hu, LY Leung, KY Tong, SM Zhang, XX Zheng. Effects of treadmill exercise on brain damage and hippocampal glutamate level after stroke in a rat model of focal cerebral ischemia. The 4th World Congress for NeuroRehabilitation. Hong Kong SAR. China. Feb 2006.

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Leung Lai Yee

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List of Abbreviations

ACA	Anterior cerebral artery
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BBB	Blood-brain-barrier
BDNF	Brain-derived neurotrophic factor
CBF	Cerebral blood flow
CCA	Common carotid artery
CT	Computed tomographic
DNA	Deoxyribonucleric acid
ECA	External carotid artery
ECF	Extracelluar fluid
fMRI	functional magnetic resonance imaging
GABA	Gamma aminobutyric acid
H&E	Hematoxylin and eosin (H&E)
HPLC	High performance liquid chromatography
ICA	Internal carotid artery
IHC	Immunohistochemistry
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
MRI	Magnetic resonance imaging
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NMES	Neuromuscular electrical stimulation
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
PCA	Posterior cerebral artery
TA	Tibialis anterior
TIA	Transient ischemic attack
TNF	Tumor necrosis factor
t-PA	tissue plasminogen activator
TTC	Triphenyltetrazolium chloride
TTX	tetrodotoxin
UV	Ultraviolet

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CHAPTER 1 INTRODUCTION

1.1 Overview of stroke

Stroke ranks as the third leading cause of death, just behind heart disease and cancer, in Hong Kong and United States (HKHA 2002, AHA 2005). It is also a leading cause of serious and long-term disabilities, which include loss of motor, sensory and cognitive functions. According to the American Heart Association, the estimated direct and indirect cost of stroke in 2005 is US$56.8 billion. Nearly half of the direct cost is spent on drugs and medical durables (AHA 2005). Stroke is expensive because so many people end up in hospital for fairly long periods of time and follow-up care is necessary for lasting deficits. The enormous medical cost arouses public's concern about these cerebrovascular accidents.

1.2 Classification of stroke

The World Health Organization (WHO) defined stroke as 'rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24h or longer, or leading to death, with no apparent cause other than of vascular origin' (Markus 2003a). There are mainly two kinds of disturbance of cerebral function – ischemia and hemorrhage. Among all stroke in the United States, 88% are ischemic whereas 12% are hemorrhagic (AHA 2005).

Ischemic stroke occurs when an artery supplying the brain with blood becomes blocked, suddenly decreasing or stopping blood flow and ultimately causing a brain infarction. The artery blockage and brain infarction are usually caused by blood

clots (NINDS 2003). This kind of stroke can be classified into global and focal ischemia. Global ischemia is commonly resulted from failure to perfuse the brain from the heart due to cardiac arrest or collapse of the systemic circulation. Focal ischemia occurs when vessels supplying to part of the brain are occluded, which leads to necrosis of neurons and other cells of that part of the brain. Since collateral flow is allowed to perfuse the brain during focal ischemia, there is rarely complete loss of cerebral blood flow. Thus, core and penumbra regions can be found only in focal ischemic brain (Vaughan and Bullock 1999). This is a major feature to distinguish between global and focal ischemia.

Hemorrhagic stroke occurs when an artery in the brain bursts, blood spews out into the surrounding tissue which upsets the blood supply and the delicate chemical balance of neurons. One common cause of this kind of stroke is a bleeding aneurysm, which is a weak or thin spot on an artery wall. Under high arterial pressure, these weak spots balloon out over time and their thin walls can rupture easily, which leads to blood spilling into the space surrounding brain cells (NINDS 2003). Hemorrhagic stroke is further divided into two sub-types, based on whether the hemorrhage involves the brain parenchyma (Intracranial hemorrhage) or involves subarachnoid space (Subarachnoid hemorrhage) (Vaughan and Bullock 1999). Figure 1.1 shows the morphological differences between hemorrhagic and ischemic stroke.

Transient ischemic attack (TIA) is a mini-stroke that lasts up to 24 hours. It starts just like a stroke but then resolves leaving no noticeable symptoms or deficits. For

almost all TIAs, the symptoms disappear within an hour. TIA is a warning sign that the person may be at risk for a more severe stroke (NINDS 2003).

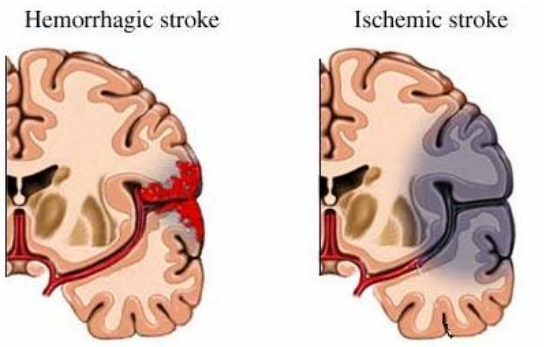
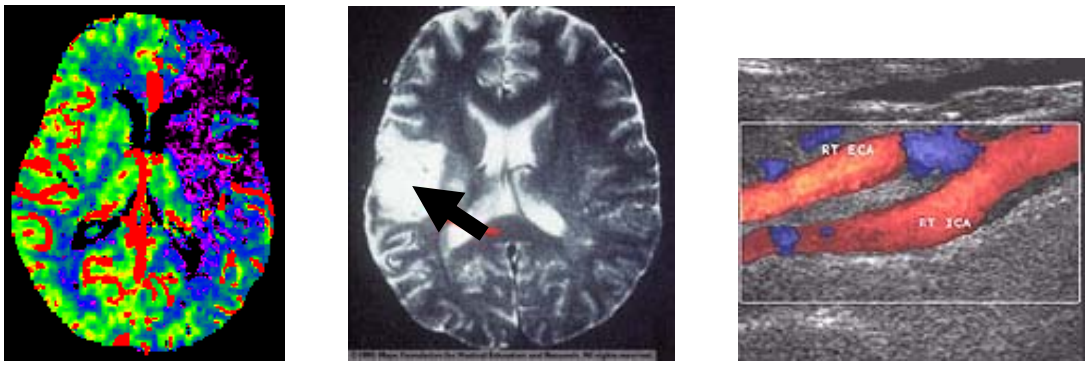


Figure 1.1 Differences between Hemorrhagic and Ischemic stroke. It can be noted that bleeding occurs inside or around the brain tissue in hemorrhagic case (Left); while blockage of blood flow to an area of the brain in ischemic case (Right) (Nucleus Medical Art 2005).

1.3 Diagnosis and Treatment

Since medical treatments for ischemic stroke and hemorrhagic stroke are different, it is necessary to distinguish between them by using imaging techniques such as computed tomographic (CT) scanning, magnetic resonance imaging (MRI) or ultrasound (as shown in Figure 1.2). CT scanning is very useful in identification of intracranial hemorrhage, which shows as a bright white abnormality against the grey background (Thomas 2000). However, MRI is preferred nowadays since its sensitivity for stroke diagnosis within 24 hours is better. It can characterize an ischemic lesion and suggest where viable tissue remains (Ebrahim and Harwood 1999). Ultrasound is also widely used since it provides a simple, relatively cheap and non-invasive method of imaging. By using Duplex ultrasound, B-mode images of the carotid arteries and Doppler information on blood flow velocity can be obtained (Markus 2000b).

Information obtained from diagnosis could be used to define the stroke pathology, so as to establish an effective therapeutic protocol for stroke patients. Treatment may include medication, surgery and post-stroke rehabilitation.



(a) (b) (c)

Figure 1.2 (a) CT Scanning of brain with infarct area (right side), in which it can be used to confirm the diagnosis of stroke and tell whether the stroke is caused by a hemorrhage in the brain; (b) MRI image of brain with infarct area (pointed by arrow), which can be used to identify and further localize the site of the stroke and find the source; (c) Ultrasound imaging of carotid artery, which can determine whether there is a blockage in an artery that carry blood to the brain. Normal blood flow is in red whereas blue areas show the blockage in the carotid artery (SIR 2004).

Drug therapy is the most common treatment for stroke and there are mainly three classes of drugs: antithrombotics, thrombolytics and neuroprotective agents. The first two drugs are used for treatment of ischemic stroke while neuroprotectants are used in both types of stroke. These agents aim at preventing cascade of events leading to ischemic neuronal damage. Antithrombotics such as aspirin and heprin are used within 48 hours of symptom onset to prevent the formation of blood clots which may block the cerebral artery. Thrombolytics agents such as tissue plasminogen activator (t-PA) and alteplase are used to dissolve the blood clot that is blocking blood flow to the brain. However, these drugs may increase the chance of symptomatic intracerebral hemorrhage. Neuroprotectants such as calcium antagonists and glutamate antagonists can protect the brain from secondary injury

caused by stroke through blocking some important pathways leading to neuronal death (Barber *et al* 2001; NINDS 2004).

Regarding the surgery treatment, carotid endarterectomy and extracranial/ intracranial bypass are two widely used surgical techniques for stroke. The former one is a surgical procedure to remove fatty deposits from the inside of one of the carotid arteries. The latter one is a procedure that restores blood flow to a blood-deprived area of brain tissue by rerouting a healthy artery in the scalp to the area of brain tissue affected by a blocked artery (NINDS 2004).

Post-stroke rehabilitation aims at reducing the handicapping effects of disease by processes of reablement and resettlement. This treatment approach involves a multi-disciplinary team which can comprise patient, family, therapists, nurses, social workers and doctors (Ebrahim and Harwood 1999). Standard motor rehabilitation after stroke typically involves a mix of approaches such as neurofacilitation techniques and task-specific training. Neurofacilitation aims at retraining motor control by promoting normal or inhibiting abnormal movement while task-specific training focuses on functional tasks, which involves the interplay of different systems of our body, especially musculoskeletal, perceptual, cognitive and neural systems (Schaechter 2004). Timing and intensity of the rehabilitation are critical factors affecting the outcome of the prescribed therapy in people after stroke. The early initiation and intensity correlate positively to better recovery after stroke (Ottenbacher and Jannell 1993; Kwakkel *et al* 1997). However, some experimental studies suggest that aggressive early intervention may significantly exacerbate the infarction of the brain (Kozlowski *et al* 1996; Humm 1998). In a standard motor

rehabilitation for acute stroke, the duration of exercise is typically 30 to 60 min per day and tends to decrease in chronic stage. The rehabilitation period depends on the degree of impairment and function deficits (Schaechter 2004). These typical conditions of post-stroke rehabilitation would result a rapid recovery of motor function during the first month after stroke, followed by a slower pace of recovery in the subsequent months and finally reaching a plateau by 6 months post-stroke (Hendricks *et al* 1999).

1.4 Objectives

The objective of this study was to investigate the neurological, functional and physiological effects of neuromuscular electrical stimulation and treadmill training in rehabilitation after ischemic stroke. Focal cerebral ischemia was induced by occluding middle cerebral artery in rats. Treadmill exercise and neuromuscular electrical stimulation were prescribed to the rats 24 hours after stroke for two weeks. *In vivo* and post-mortem studies were carried out. This study may provide more insights in the mechanism of motor recovery through these rehabilitative interventions. It could definitely be a strong foundation for improving existing rehabilitation strategy using neuromuscular electrical stimulation and treadmill training for people after stroke.

CHAPTER 2 LITERATURE REVIEW

2.1 Pathophysiology of Stroke

The development of successful therapeutic strategies is resulted from a better understanding of the pathophysiology of stroke (Dirnag *et al* 1999). The pathophysiology of cerebral ischemia is illustrated in a simplified way in the following section so as to give a whole portrait of the processes involved. Briefly, cerebral ischemia results in edema, damage and malfunctions of organelles (mitochondria), DNA damage and inflammation, increase in intracellular calcium ions concentration and extracellular glutamate levels. All these results contribute to neuronal cell death, either through apoptosis or necrosis. Pathophysiology of stroke provides insights for researchers to develop novel treatments.

2.1.1 Ischemia and energy depletion

Energy was necessary for maintaining ionic gradients and supporting synthetic and catabolic activity in neurons and glial cells (Dirnag *et al* 1999). Reduction in cerebral blood flow (CBF) due to cerebral ischemia would decrease the oxygen and glucose supplying to the brain, and thus lead to energy depletion. This results in failure of ion pumps. Dramatic changes in ion fluxes is always the first step to damage brain cells. Besides, energy depletion causes mitochondrial injury, leukocyte activation, inflammation, oxygen radical generation and excitotoxin release. These result in cascade of events leading to neuronal death (Traystman 2003). Figure 2.1 shows the potential mechanisms of injury from ischemia.

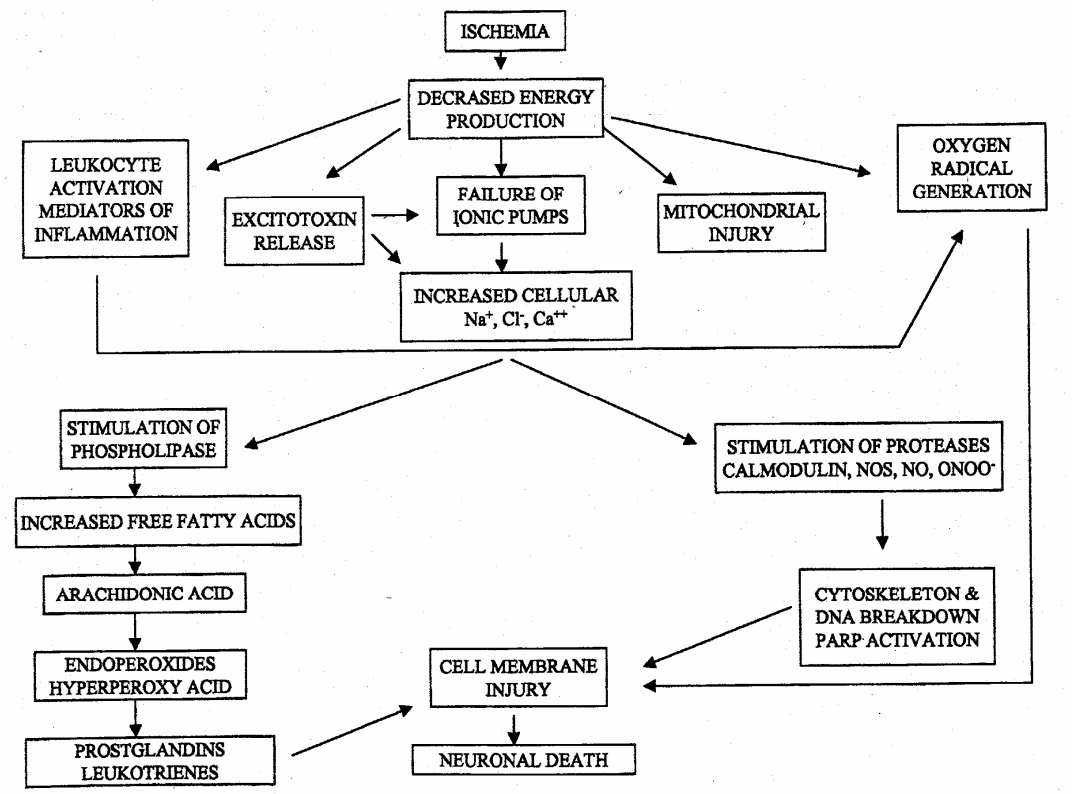


Figure 2.1 Potential mechanisms of injury from ischemia (Traystman 2003)

2.1.2 Failure of ionic pumps

Failure of the sodium-potassium ATPase and other energy-dependent ionic pumps disable the maintenance of ionic gradients across the membrane of neurons and glial cells. Sodium ions and calcium ions accumulate within the cell whereas there is an efflux of potassium ions from cells to extracellular space (Iadecola 1999). The cell membrane then depolarizes due to the changes in ionic fluxes. Increase in extracellular potassium concentrations leads to massive transmitter release, which triggers ionic fluxes through voltage-dependent and agonist-operated channels (Kristian and Siesjo 1997). Increase in transmitter such as glutamate in extracellular space causes sodium and chloride entering the neuron and glial cells via channels for monovalent ions (Dirnag *et al* 1999). At this stage, there is further increase in

extracellular potassium ions and uptake of calcium, sodium and chloride ions. Figure 2.2 shows the pathway of the above processes. Since concentration gradient is developed, in which influx of sodium and chloride ions is much greater then efflux of potassium ions, water tends to move into the cells by osmosis (Dirnag *et al* 1999). Inflow of ions and water finally leads to edema, as shown in Figure 2.3 and if these processes are prolonged, edema may cause cell lysis and death.

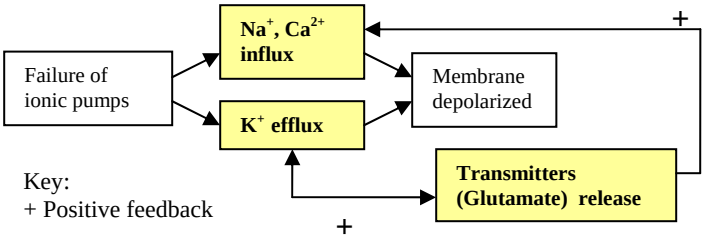


Figure 2.2 Increase in Na^+ and Ca^{2+} influx and efflux of K^+ due to failure of ionic pumps

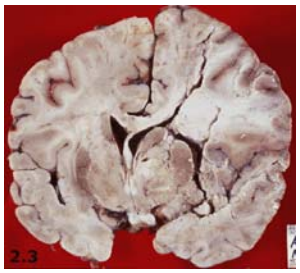


Figure 2.3 Edema of the right hemisphere after cerebral ischemia and early disintegration in the infarcted tissue (Akron Children's hospital 2004)

2.1.3 Oxygen radical generation

Generation of oxygen free radicals is triggered by calcium ions influx into neurons and glial cells during ischemia. Concentration of intracellular calcium ions increases in all neuron and glial cells, but with a greater extent in vulnerable cells such as CA1 region of hippocampus. This initiates a cascade of destructive metabolic processes, which lead to death of the neuron during cerebral ischemia. These processes include activation of proteolytic enzymes and increasing nitric oxide (NO) production by neuronal nitric oxide synthase (nNOS). The proteolytic enzymes degrade cytoskeletal proteins and extracellular matrix protein (Dirnag *et al* 1999). NO causes the inhibition of mitochondrial respiration, glycosis, and DNA replication, which initiates ischemic brain damage. It reacts with superoxide to yield

peroxynitrite ($ONOO^-$). This highly toxic anion is sufficiently stable to diffuse over several cell diameters to critical cellular targets such as mitochondria, to cause damages. Moreover, NO facilitates neurotransmitter release or inhibits its uptake in neurons including dopamine, acethylcholine and glutamate (Dalkara and Moskowitz 1997). Thus, excessive glutamate is accumulated in extracellular space, which causes a cascade of reaction as described previously. Hydroxyl radical is produced and it causes protein oxidation and damages DNA in cells (Chan 1999). NO damages DNA structures by several processes including DNA nitration, deamination and oxidation. It inhibits DNA synthesis by depressing ribonucleotide reductase activity. DNA damage by free radicals also triggers apoptosis whereas the structural damage of the cells leads to neuronal necrosis. Inducible NOS (iNOS) is expressed in microglia and it invades inflammatory cells, in which increase in NO levels sustains 24-27 hours after the induction of ischemia. Other reactions mediated by NO are shown in Figure 2.4.

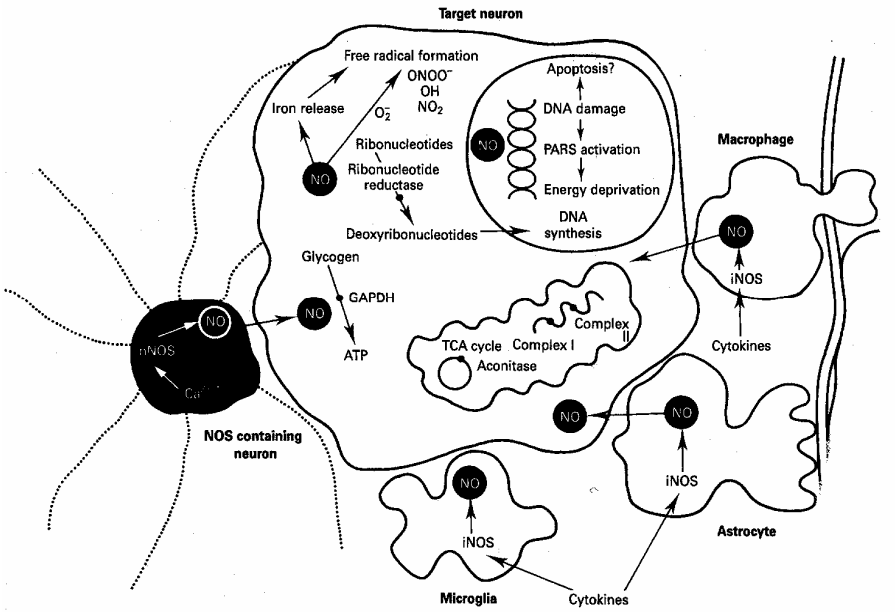


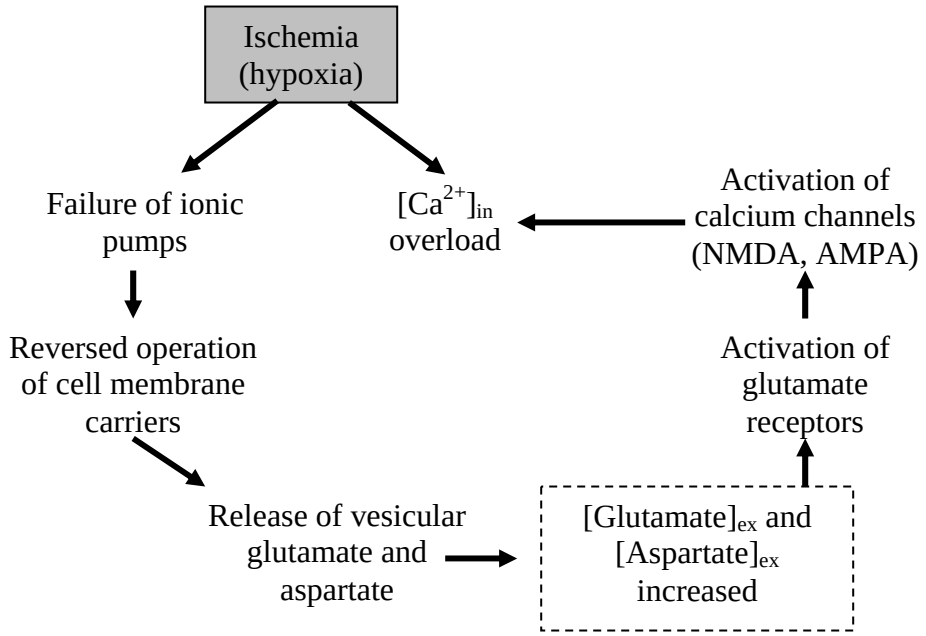
Figure 2.4 NO-mediated neurotoxicity. NO is generated in vascular endothelium and NOS-containing neurons at the onset of ischemia (Dalkara and Moskowitz 1997)

2.1.4 Excitotoxin release

Glutamates are excitatory neurotransmitters, which belong to the family of amino acids. They are released from vesicles when depolarization occurs. Figure 2.5a illustrated the proposed mechanism of ischemia-induced glutamate release. Due to the failure of the ion pumps in cerebral ischemia, the cell membrane is depolarized. This stimulates the release of glutamates from the vesicles into the synaptic cleft. A normal neuron would usually take the released glutamate back into the cell rapidly by energy-dependent mechanisms. However, ischemic neuron fails to do so because of energy crisis. The elevation of extracellular glutamate level causes a prolonged and excessive activation of membrane glutamate receptors (Kristian and Siesjo 1997) and thus activates of receptor-mediated calcium channels such as NMDA and AMPA propionic channels (Fisher 1999). The opening of these channels leads to inflow of calcium ions, which may lead to delayed neuronal death.

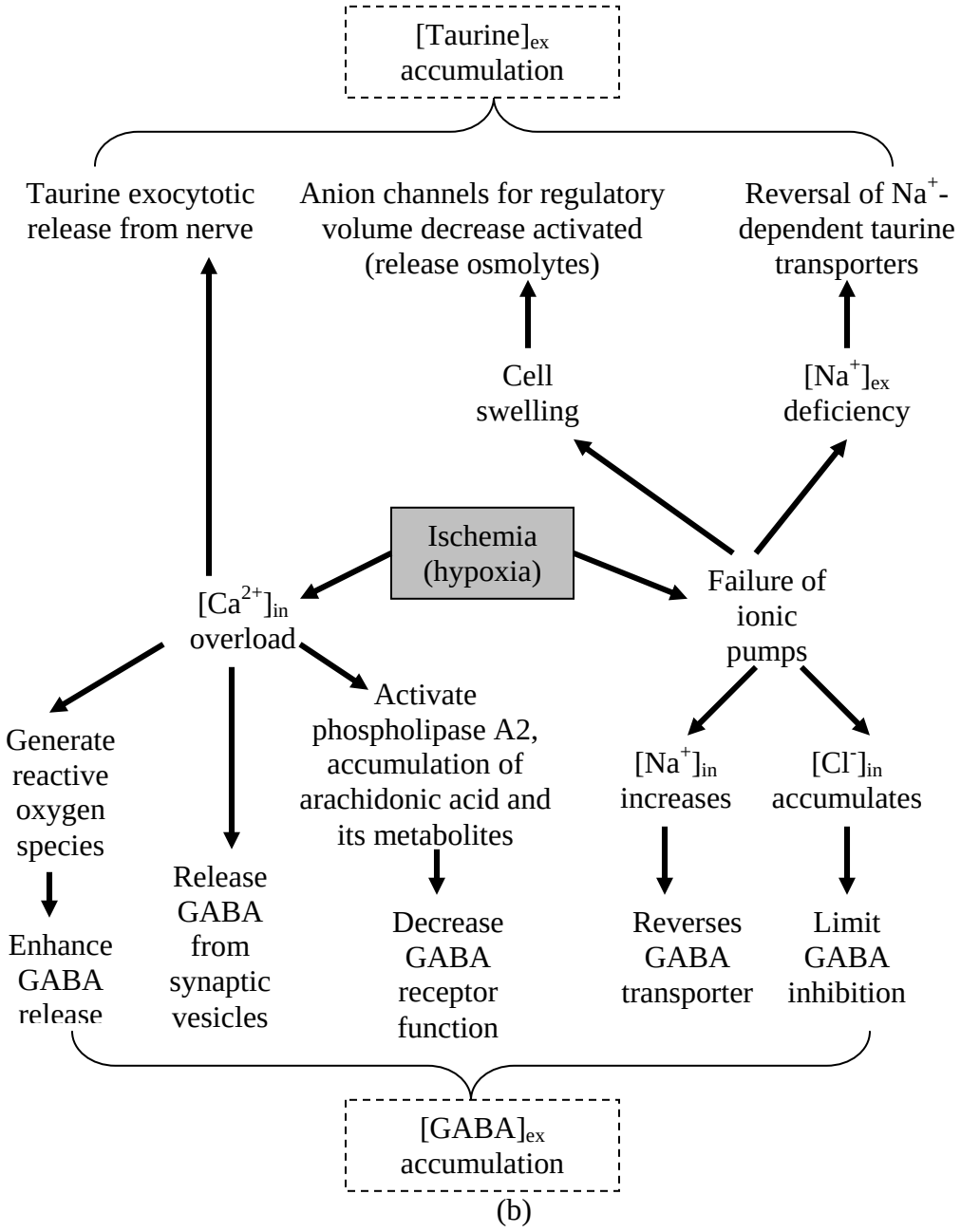
Other neurotransmitters in the family of amino acids, such as aspartate, taurine and GABA, also play an important role in ischemic injury. Similar to glutamate, aspartate is a major excitatory neurotransmitters in the brain and its excitotoxicity contributes to neuronal death in cerebral ischemia (Purves 2001). Much of the emphasis has been centered in ischemia-induced glutamate. Due to the equivocal identification of these two excitatory amino acids (Siegel et al 1999), it was assumed that they might have similar mechanism of being evoked by ischemia, as demonstrated in Figure 2.5a. Taurine and GABA, the abundant inhibitory neurotransmitters in the brain, released simultaneously with the excitatory amino acids (Saransaari and Oja 1997). The mechanism of ischemia-evoked release of

taurine and GABA was illustrated in Figure 2.5b. They may act as neuroprotectants against the excessive release of excitatory amino acids (glutamate and aspartate). They maintain the homeostasis in the brain upon hyperexcitation by increasing the conductance of chloride ions and thus inducing hyperpolarization and reducing cell excitability. Taurine itself could attenuate calcium ions influx and thus, preventing the damaging cascade by calcium overload (Saransaari and Oja 1998). The elevated levels of these amino acids returned to near baseline levels within 60min after onset of ischemia (results from literatures were presented in latter section in this chapter).



(a)

Figure 2.5 Ischemia-induced (a) Glutamate and Aspartate accumulation (Kristian and Siesjo 1997). *Key: $[X]_y$ denotes as concentration of amino acids X, subscript 'y' indicated intracellular 'in' and extracellular 'ex'.*



(b)

Figure 2.5 (cont') Ischemia-induced (b) GABA and Taurine accumulation (Schwartz-Bloom and Sah 2001; Phillis and O'Regan 2003; Saransaari and Oja 2000). *Key: $[X]_y$ denotes as concentration of amino acids X, subscript 'y' indicated intracellular 'in' and extracellular 'ex'.*

2.1.5 Mitochrondrial injury

The primary effect of ischemia on mitochondria is the inhibition of mitochondrial oxidative phosphorylation due to lack of oxygen. This inhibition reduces the

production of ATP and enhances anaerobic metabolism. Blockage of oxidative metabolism leads to insufficient energy to maintain the membrane potential required for driving Ca^{2+} uptake into the mitochondria (Morley *et al* 1999). This disturbance of the normal function of mitochondria also leads to increase in mitochondrial free radicals production. These free radicals produced during ischemia disrupt the inner membrane of mitochondria and the leaky membrane promotes swelling of the mitochondria, cessation of ATP production and oxygen free radicals burst (Dirnag *et al* 1999). Besides, the change in membrane permeability transition causes the release of protein apoptosis-inducing factor from the intermembrane space of the mitochondria (Ahmed *et al* 2001).

2.1.6 Leukocyte activation, mediators of inflammatory

Calcium-related activation of intracellular second messenger systems and increase in oxygen free radicals and hypoxia trigger the expression of a number of proinflammatory genes of injured cells. Injured brain cells thus produce mediators of inflammation such as platelet-activating factors and tumor necrosis factor alpha (TNF-α) which induce expression of adhesion molecules on the endothelial cell surface (Dirnag *et al* 1999). Leukocytes contribute to stroke damage by entering infarct tissue and exacerbating cell death through cytotoxic interactions, or by reoccluding vessels after reperfusion so as to reduce cerebral blood flow. Leckocytes damage tissues by producing proteases, phospholipases, nitric oxide, hydrogen peroxide and superoxide (Danton and Dietrich 2003). Other inflammatory mediators also contribute to the brain damage during or after ischemia. For example,

one of the common mediators, arachidonic acid derivatives worsens the damage by increasing cerebral edema and triggering irreversible neuronal damage (Danton and Dietrich 2003).

2.2 Animal models of cerebral ischemia

The animal models have provided profound knowledge regarding the cerebral ischemia and evidence to support the efficacy of treatment strategies such as neuroprotective agents and surgery. The animal models for researches of cerebral ischemia usually fall into two main categories, global and focal cerebral ischemia. Global cerebral ischemia could occur when cerebral blood flow (CBF) in most or all of the brain is reduced (Traystman 2003). This widespread reduction or cessation of blood flow causes selective neuronal death (primarily in hippocampus and striatum). This model is related to cardiac arrest and coronary artery bypass graft surgical interventions (Campbell and Hunter 1999). Whereas, focal cerebral ischemia could occur when CBF is reduced at a very distinct and specific region of the brain (Traystman 2003). This local disturbance of blood flow could lead to either selective neuronal death (transient focal ischemia) or pannecrosis (induced ischemia lasts for more than 30min) (Li and Fisher 2001). Pannecrosis refers to total death of all types of cells and the selective neuronal necrosis means death of neuronal cells which are vulnerable to attack, for example, cells in the hippocampus. Figure 2.6 shows the main arteries supplying the brain from the heart (from Aortic arch up to anterior communicating artery). Figure 2.7 shows the location of different area of

the human brain. These would help to have a better understanding in different occlusion methods which would be discussed in the following sections.

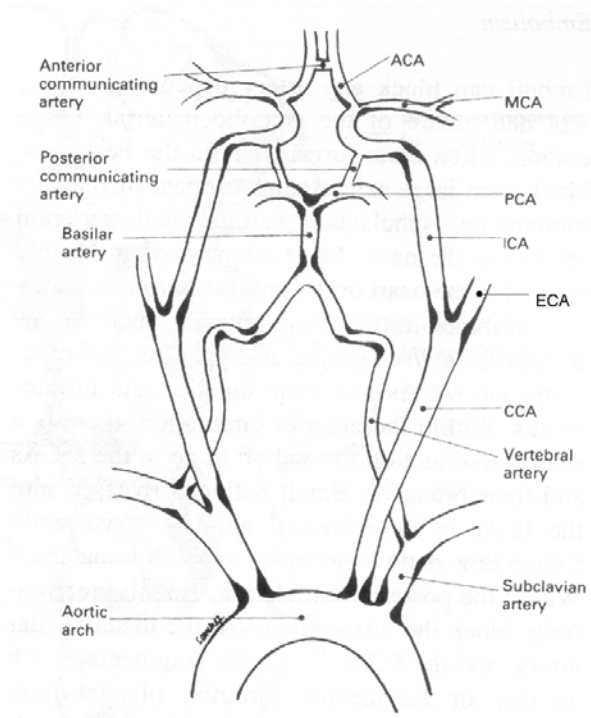


Figure 2.6 Main arteries supplying the brain from the heart (ACA: anterior cerebral artery; CCA: common carotid artery; ICA: internal carotid artery; ECA: external carotid artery; MCA: middle cerebral artery; PCA: posterior cerebral artery) (Caplan 2000a)

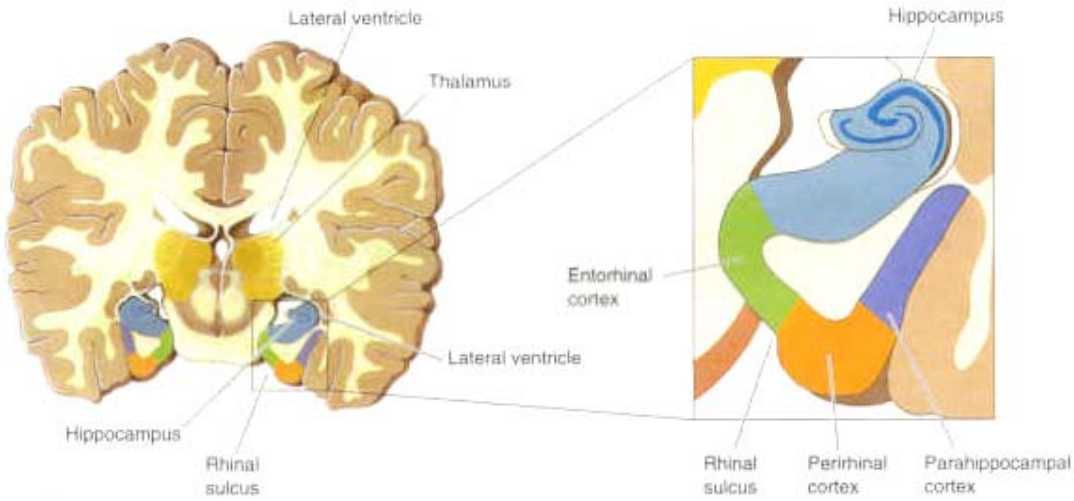


Figure 2.7 Coronal section of the human brain: Lateral ventricle, thalamus, rhinal sulcus, hippocampus, rhinal sulcus, perirhinal cortex, parahippocampal cortex (Gottfried 2000).

2.2.1 Global cerebral ischemia models

This model is usually used for investigating biochemical, metabolic and physiologic responses after reduction of CBF. It is also used to study the mechanisms regarding the process of selective neuronal vulnerability in hippocampus after chronic survival. There are several methods to induce global cerebral ischemia in animals, mainly including bilateral carotid artery occlusion, two-vessel occlusion, four-vessel occlusion, decapitation and neck tourniquet (Traystman 2003; Green and Cross 1997).

2.2.2 Focal cerebral ischemia models

Focal cerebral ischemia models are widely used in stroke related researches due to their strong resemblance to human thromboembolic stroke. They are usually used for studying pharmacological neuroprotection, mechanisms of ischemic injury and for characterization of genes and proteins involved after stroke. Middle cerebral artery (MCA) is the most commonly affected vessel in stroke (Green and Cross 1997). Therefore, cerebral ischemia is commonly induced in animal by occluding MCA. This occlusion leads to reduced CBF in both striatum and cortex. The severity and extent of damage depend on duration of occlusion, site of occlusion along the MCA (proximal or distal) (Figure 2.8), and the amount of blood flow into the MCA territory (Traystman 2003). The cerebral blood flow can be blocked by five different methods including injection of embolus, induction of thrombosis photochemically, injection of vasoconstrictor, direct ligation and insertion of intraluminal suture.

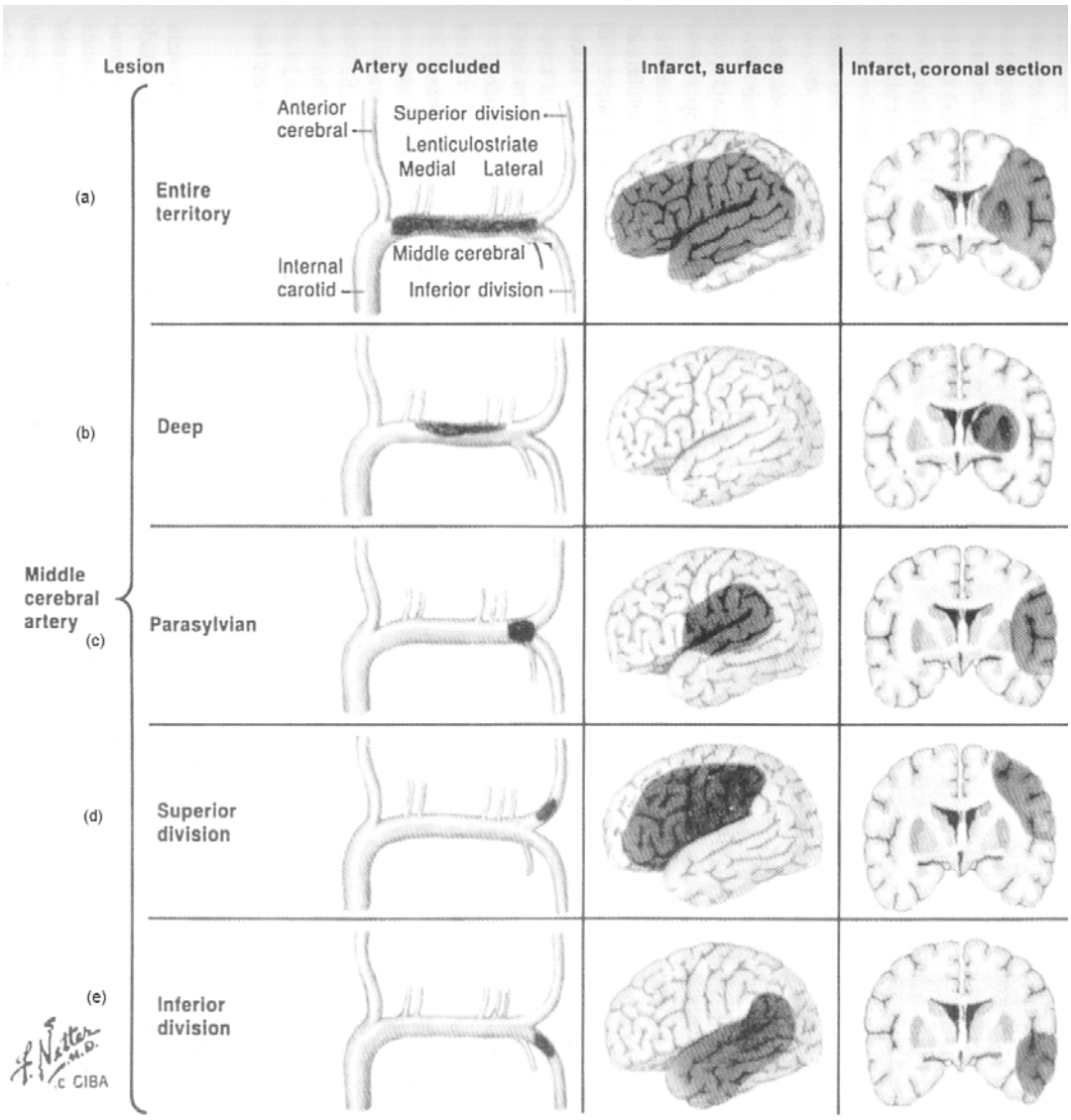


Figure 2.8 Patterns of occlusion of the middle cerebral artery and their anatomic correlates (Caplan 2000b)

2.2.2.1 Embolus model

Homologous blood clot fragments or emboli are injected directly into the carotid artery via a retrograde catheter. The emboli can be carbon or plastic microspheres, silicone cylinders or simply air (Traystman 2003). Severity of damage depends on number and size of emboli injected. Since the emboli usually flow into different branches of the vessels once they are injected, the location of damage is not

consistent which results in diffused distribution of infarction (Li and Fisher 2001). This explains why this model cannot be used for investigating mechanisms of ischemic injury and therapeutic interventions. However, since it highly resembles to human ischemic stroke, it is very useful in examining thrombolytic agents and showing protective effect of tissue plasminogen activator (tPA), which is an acute treatment for people after stroke (Green and Cross 1997).

2.2.2.2 Photochemically induced thrombosis model

By injecting photosensitizing dye such as rose Bengal or photofrin into several branches of distal MCA, combined with irradiation with a light beam at a specific wavelength, consistent infarction in the frontoparietal neocortex can be induced (Traystman 2003). Free radicals are generated by the reaction between the circulating dye and light. These free radicals lead to platelet aggregation and thrombosis. The location and extent of lesions are determined by selectively illuminating the brain tissue using different intensities of light and different doses of dye. Small or no penumbral region is noted. Vasogenic edema and breakdown of blood-brain-barrier (BBB) are occurred rapidly. This model is rarely used to test neuroprotective agents since the pathophysiologic processes induced are not directly relevant to those in human. However, it is useful in investigating some of the neurochemical changes that occurred in core region after focal cerebral ischemia (Li and Fisher 2001; Green and Cross 1997).

2.2.2.3 Endothelin-induced middle cerebral artery occlusion model

Endothelin-1 exhibits vasoconstrictor effect. Researchers made use of this effect to reduce the CBF by applying Endothelin-1 to MCA. This model is not suitable in drug development or evaluation since the duration of ischemia is not controllable and the damage is varied. This is due to different vessels have different responses to Endothelin-1 and the vasoconstriction induced may disappear gradually (Li and Fisher 2001).

2.2.2.4 Direct surgical middle cerebral artery occlusion model

After sub-temporal craniectomy, the MCA can be occluded by direct ligation, coagulating, clipping or snaring its trunk or branches. Reperfusion can be established by pulling or releasing the snare or clips (Li and Fisher 2001). Occlusion results in infarction of the cortex and caudoputamen areas. The neuropathological outcomes are greatly affected by the site and extent of the occlusion. This model requires more experience and skills due to variations of MCA anatomic pattern among individuals. Moreover, direct exposure of brain by craniectomy could change intracranial pressure and blood-brain-barrier permeability (Li and Fisher 2001).

2.2.2.5 Intraluminal suture middle cerebral artery occlusion model

Cerebral ischemia induced by intraluminal suture is achieved by inserting a 4-0 or 3-0 nylon suture (depending on the size of the animals) through either common carotid artery (CCA), external carotid artery (ECA) or internal carotid artery (ICA). The suture is advanced cranially to occlude the collateral circulation from the

anterior communicating arteries and to block the MCA. Its length inserted (measured from the bifurcation of CCA, usually ranged 17-22mm) depends on body weight, size of the suture tip and the location of bifurcation of CCA. Damage is produced at lateral caudoputamen and frontoparietal cortex. To make the suture more adherent to the surrounding endothelium, poly-L-lysine can be coated on the suture. Transient or permanent ischemia can be easily performed by withdrawing or maintaining the suture. This model is easy to perform and does not require craniectomy. Moreover, compared with the models involving craniectomy, physiologic complications caused by the surgery are minimal (Traystman 2003; Li and Fisher 2001). In Table 2.1, the advantages and disadvantages of different models are revealed.

Table 2.1 Advantages, disadvantages and features of different models of focal cerebral ischemia models (Campbell and Hunter 1999; Green and Cross 1997)

Models	**Advantages**	**Disadvantages**	**Features**
Embolic	Simple, No craniectomy	No control over the distribution of microspheres No uniformity of the size or location of the infarcts	For examining thrombolytic agents
Photo -thrombotic	No craniectomy Proved to be useful in examining some of the neurochemcial changes that occur in core region Can induce permanent or transient ischemia Possible to implant arterial and venous cannulas for infusion studies in both anesthetised and conscious animals	Breakdown of Blood-brain-barrier Little collateral blood flow Pathology not demonstrated to be represetnive of thrombotic stroke in humans Time consuming for transient occlusion, as surgery for transient ischemia is complex	No penumbra area produced
Endothelin-1	Simple, minimally invasive surgery, applicable to rapid screening of potential neuroprotectanats Reliable measurement of damage	Skilled manipulation required to place cannula in correct position Some variability with wearing off of the endothelin, which may make time-window studies harder to carry out	Selective neuronal damage
Direct surgical	Higher long-term survival	Extensive surgical training required	Penumbra area may be smaller
Intraluminal	Simple surgery, allowing rapid screening of potential neuroprotectants Permanent or transient occlusions easily performed Possible to implant arterial and venous cannulas for infusion studies in both anesthetized and conscious animals Reliable measurement of damage	Need to visualize position of filament either in situ postmortem or by staining of endothelium for transient occlusions	Ischemic core area and penumbra area are produced

2.3 Assessment methods in animal stroke models

2.3.1 Neurological deficits

2.3.1.1 Postural reflexes test

Bederson *et al* examined the reliability and predictability of the direct middle cerebral artery occlusion (MCAO), which has been developed early in 1975 by Robinson and his colleagues. Forelimb flexion could be tested by holding the rats gently by the tail and by suspending it 1 m above the floor. Focal cerebral ischemic rats usually flexed their forelimb as shown in Figure 2.9, which was contralateral to injured hemisphere, whereas normal rat extended both forelimbs towards the floor. It was found that there were correlated changes in neuralgic status with the size and location of area of infarction (Bederson *et al* 1986). A relatively non-invasive cerebral ischemic rat model using intraluminal technique and a neurological examination was later established by Longa *et al* (Longa *et al* 1989), which was similar to that developed by Bederson. The neurological deficits could be graded according to their postural reflexes as listed in Table 2.2. These two scales were very similar and were widely used to assess the success of MCAo and to measure the effects of drugs (Li and Fisher 2001).

Figure 2.9 Rat with focal cerebral ischemic flexed forelimb (circled)

Table 2.2 Bederson's and Longa's neurological examination scoring scales (Bederson *et al* 1986; Longa *et al* 1989)

Score	Deficits (Bederson's scale)	Deficits (Longa's scale)
0	Normal: no observable deficit	No neurologic deficit
1	Moderate: Forelimb flexion	Failure to extend forepaw on the affected side fully
2	Sever, decreased resistance to lateral push (and forelimb flexion) without circling	Circling to the affected side
3	Same behavior as grade 2, with circling	Falling to the affected side
4	-	Did not walk spontaneously and had a depressed level of consciousness

2.3.1.2Limb placing

This assessment was originally designed by De Ryck *et al* (De Ryck *et al* 1989). They established a noninvasive photochemical stroke model and examined sensorimotor integration in forelimb placing responses to visual, tactile and proprioceptive stimuli by using this assessment. The assessment was divided into three parts based on different stimulation to the rat, and the details were summarized in Table 2.3. Figure 2.10 demonstrated the tactile and proprioceptive deficits in a rat with cerebral ischemia on the right hemisphere. It was noted that when whisker and chin contact were allowed, the contralateral forelimb placing deficit was apparently absent (Figure 2.10a). However, when turning the rat's head 45^{o} upwards to prevent the cephalic contact stimuli, forelimb placing deficit appeared (Figure 2.10b). In Figure 2.10c, the contralateral forelimb slipped off the edge of the platform as snout and whiskers lost contact with the surface. Propriceptive placing deficit of the contralateral hindlimb can be observed whereas the forelimb placing deficit was masked by cepthalic contact stimuli (Figure 2.10d).

Table 2.3 (a) Limb placing test and scoring system (De Ryck *et al* 1989)

	Limb placing test
Stimulation	**Description**
Visual	Slowly lowering the rat toward a table top and held it 10cm above the table with free hanging forelimbs. By moving the rat laterally toward the table edge, lateral as well as forward visual limb placing could be assessed
Tactile	Lightly contacting the table edge with the dorsal or lateral aspect of a rat's paw respectively
Proprioceptive	Pushing the rat's paw against the table edge to stimulate limb muscles and joints
	Put the rat along the edge of an elevated platform. Gently pulling its paw down and away from the platform edge and upon sudden release, retrieval and placing can be checked

Table 2.3 (b) Scoring system for limb placing test (De Ryck *et al* 1989)

	Scoring system
Scores	**Observation**
0	No placing
1	Incomplete and/or delayed (> 2sec) placing including interspersed flailing
2	Immediate and complete placing

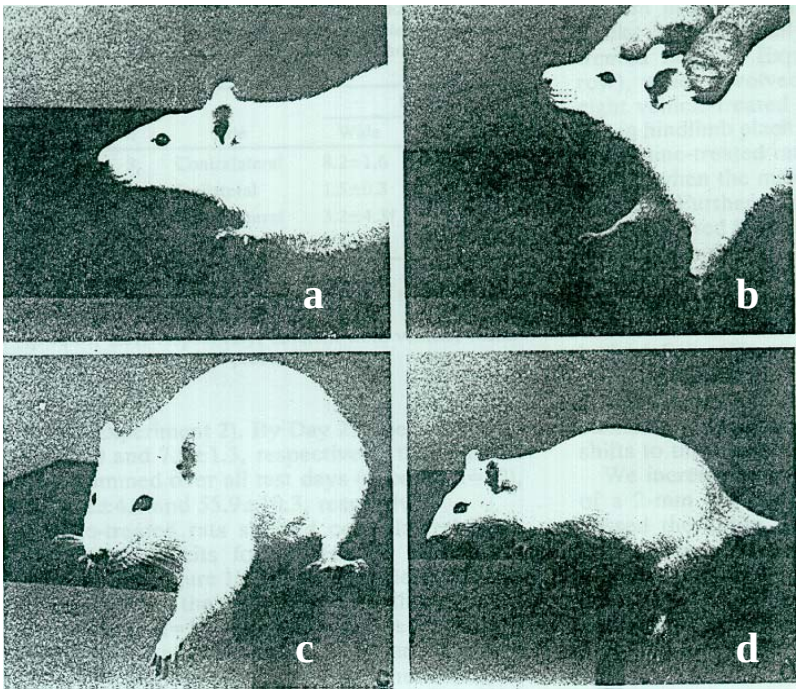


Figure 2.10 Tactile and proprioceptive limb placing deficits contralateral to the infact of the right sensorimotor neocortex (De Ryck *et al* 1989)

2.3.1.3 Walking test and rotarod

Walking and balancing abilities can be assessed by using beam walking test or grid walking test. In beam walking, the rats were trained to run from one end of a rectangular wooden beam (as shown in Figure 2.11a) to the other using adverse stimuli. The width of the beam varied between different laboratories, usually it is just enough for a normal rat to run through without slipping their limbs at the side edges. Two variables are obtained from this test, one is the time to run through the length of the beam and another one is the net slips count. The net slip count is the difference between the number of forelimb slips on contralateral side (to cerebral ischemic side) and that on ipsilateral side. The net slips count can be then calculated as a percentage of the total number of step taken as the animal ran along the beam. Figure 2.11b demonstrates a grid walking test to assess the walking ability of a rat after ischemic insults. The time to run from one end to the other and number of forepaw slips are measured (Aronowski *et al* 1996).

Rotarod assesses the balance and coordination of the rat with cerebral ischemia. It consists of a rotating drum, in which the mice or rats are placed individually on the revolving drum as shown in Figure 2.11c. Once they are balanced, the drum is accelerated and the time at which each animal fell from the drum is recorded using a stopwatch (Hunter *et al* 2000).

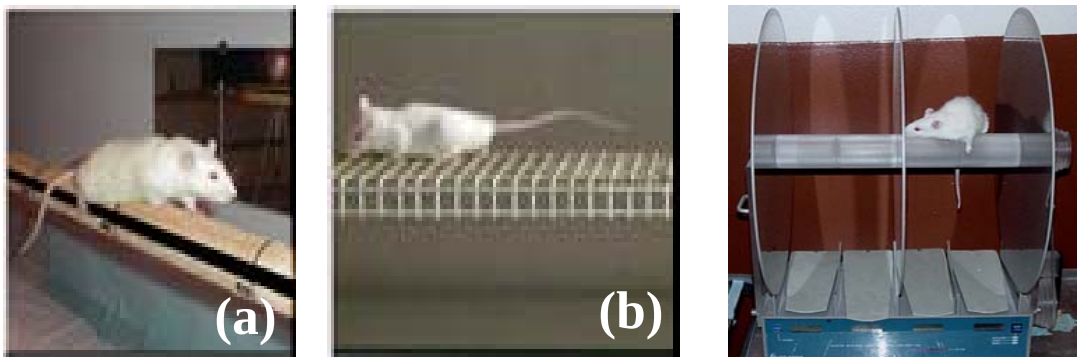


Figure 2.11 Rat performing walking and balance tests with (a) beam walking, (b) grid walking, (c) rotarod (Neurofit 2004; Medical College of Geogia 2004a)

2.3.2 Behavioral and cognitive assessments

2.3.2.1 Water maze

Morris water maze has been widely used to examine the cognitive status (spatial learning and memory) of rats with cerebral ischemia. It consists of a white circular pool with about 1.5m diameter, a platform and a video camera which is connected to a computer with tracking system (Cain and Boon 2003). The set up of the water maze is shown in Figure 2.12. The animal, usually rat or gerbil, is placed in the pool and must find a platform hidden below the water surface (made opaque with milk or paint) using visual cues in room which remain constant (Corbett and Nurse 1998). The digital files of trials from video camera were objectively analyzed for obtaining two parameters – hidden platform search time and heading error, which are defined as an instance of swimming away from the visible platform (Cain and Boon 2003). Performance in the Morris Water Maze is acutely sensitive to manipulations of the hippocampus. Incapability in finding the platform indicates impairment in memory, which can be measured as an increased latency to reach the platform. Water maze test in gerbil have been shown to distinguish between drug treated and non-treated

groups after ischemic insult in some cases (Corbett and Nurse 1998). Figure 2.13 shows the temporal change of the mean swim time and heading errors of the stroke and sham rats.

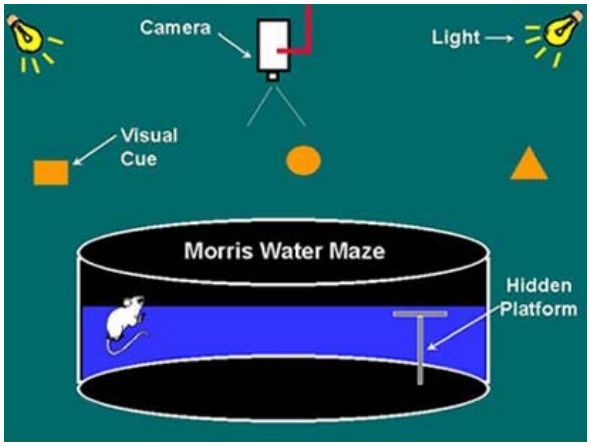
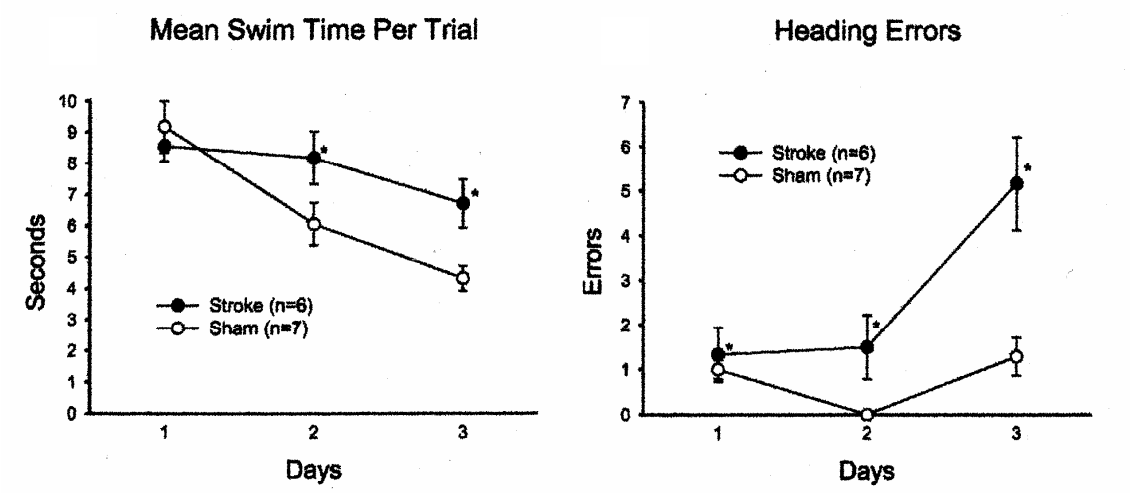


Figure 2.12 Set-up of Morris Water Maze. The visual cues can be anything in the surrounding such as posters, cabinets, doors (Medical College of Georgia 2004b).



(a) (b)

Figure 2.13 Morris water maze test with stroke and sham rats (a) Group mean swim time per trial and (b) group mean heading errors. Rats with stroke had longer swim times and made more heading errors in swimming to the visible platform (Cain and Boon 2003).

2.3.2.2 Radial arm maze

Radial arm maze is similar but more complex than water maze testing. The rat or gerbil is put at the central area, which provides access to eight arms which radiate

off the centre, as shown in Figure 2.14. Only four arms out of eight are baited with food. Once the animal learns which arms are baited, the most efficient strategy is to visit only these four arms so as to get the food rewards. Reference memory is represented by the ratio of entries into baited arms versus non-baited arms whereas revisiting an arm, in which the arm is not re-baited during the trial. This memory ability assessment has been used to provide evidence for neuroprotection following ischemia (Liang *et al* 1997).

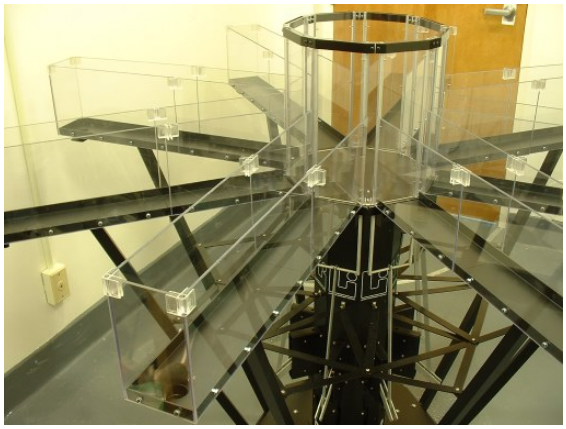


Figure 2.14 Radial arm maze (Medical College of Georgia 2004b)

2.3.3 Brain slice

Postmortem tissue analysis has been a useful technique to investigate the brain damage and to quantify the extent of the damage. It can be performed by 2,3,5-triphenyltetrazolium chloride (TTC) staining or by hematoxylin and eosin (H&E) staining, in which lesion volume after cerebral ischemia can be obtained. The lesion volume is calculated by multiplying the lesion area by the slice thickness and it is usually used as an index of the severity of ischemia damage. There are two lesion volumes being considered - direct volume and indirect volume. The former one is

obtained by summing the volumes of the lesion regions within the coronal slices whereas the latter one is obtained by subtracting the volume of the ipsilesional hemisphere from the volume of the contralesional hemisphere. The indirect lesion volume can compensate for brain edema (Li and Fisher 2001), since brain edema could increase the volume of the affected size and the overall infarction volume will be overestimated.

2.3.3.1 2,3,5-Triphenyltetrazolium chloride (TTC) staining

TTC staining provides a convenient and reliable way for evaluating infarct volume in experimental ischemic stroke. TTC staining can be done by using a 2% solution of TTC in normal saline to react with brain slices for 30 minutes at 37^{o}C (usually in the incubator). Normal tissue stains red while ischemic tissue does not stain, which is white in colour (Figure 2.15). The validity of TTC staining is based on mitochondrial enzyme damage. Red colour in normal tissue is due to the reduction of TTC by mitochondrial enzymes. The mitochondria are damaged in ischemic tissue, therefore no reaction of TTC occurs and it results in white colour. This technique was useful between 6 to 72 hours after the onset of ischemia. However, it might not accurately reflect the ischemic damage when stained earlier than 6 hours since destruction of mitochondrial enzyme requires time. After 72 hours, the infarct volume obtained based on TTC staining may be underestimated due to proliferative inflammatory responses which may obscure the periphery of ischemic damage (Li and Fisher 2001). It is preferable to stain the brain as soon as the animal dies in order to obtain valuable and accurate results. Delayed TTC staining within 8 hours

after animals' deaths would still be useful since mitochondrial enzyme degrade slowly after death (Li *et al* 1997).

2.3.3.2 Hematoxylin and Eosin (H&E) staining

H&E staining is a traditional technique to identify some cell types according to their size, shape and specific locations. In cerebral ischemia studies, it is used for evaluating ischemic changes that may include both acute-type and delayed-type damage. Cellular swelling, neuropil spongiosis (vacuolation), and shrunken neurons are obvious features in acute-type damage after ischemia, whereas eosinophilic neurons, which are irreversibly damaged, are prominent in delayed-type damage. (Li and Fisher 2001). Necrotic injury identified in H&E stained sections was characterized by nuclear pyknosis, karyorrhexis, or karyolysis which contain dispersed chromatin clumps, associated with increased cytoplasmic eosinophilia (red neurons) and nuclei lacking cellular structures (ghost neurons). Apoptotic cells were characterized by plasmalemma sealing which produced membrane-bound apoptotic bodies of roughly spherical or ovoid shape. Cells containing more than two apoptotic bodies, which are typically intensely dark purple-blue, were referred to as apoptotic cells (Li *et al* 1998). The morphological features of intact, injured, necrotic and apoptotic neurons in the H&E stained brain sections are illustrated in Figure 2.16.

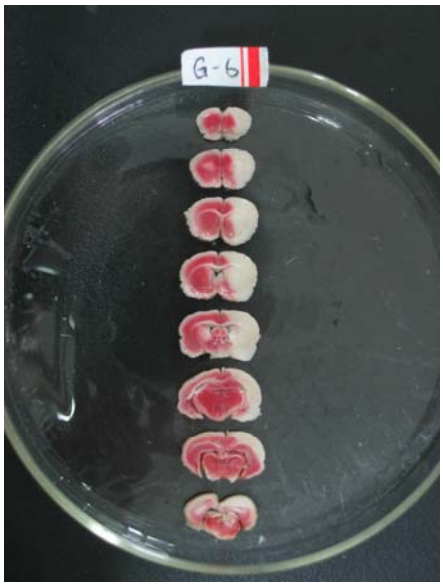


Figure 2.15 TTC stained brain slices of a rat with focal cerebral ischemia induced by Intraluminal MCAo

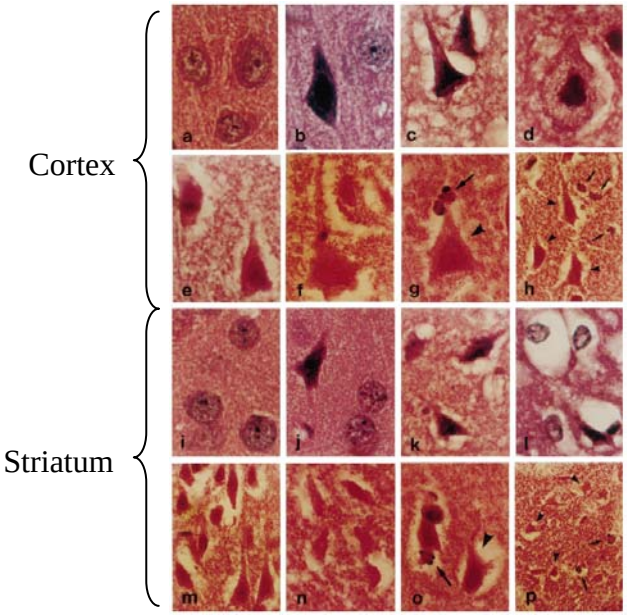


Figure 2.16 Photomicrographs show structural features of intact, injured, necrotic and apoptotic neurons on H&E preparation in rats 2 days after 2 h of MCAo. a–b, i–j: contralateral hemisphere; c–h, k–p: ischemic core. a,i: intact neurons; b,j: non-scalloped shrunken dark neurons; c,k: scalloped shrunken dark neurons; d,l: swollen neurons; e,m: red neurons; f,n: ghost neurons; g–h, o–p: necrotic (arrow heads) and apoptotic (arrows) cells. a–g, i–o, x 400; h,p, x200 (Li *et al* 1998)

2.3.4 Neurochemical aspects

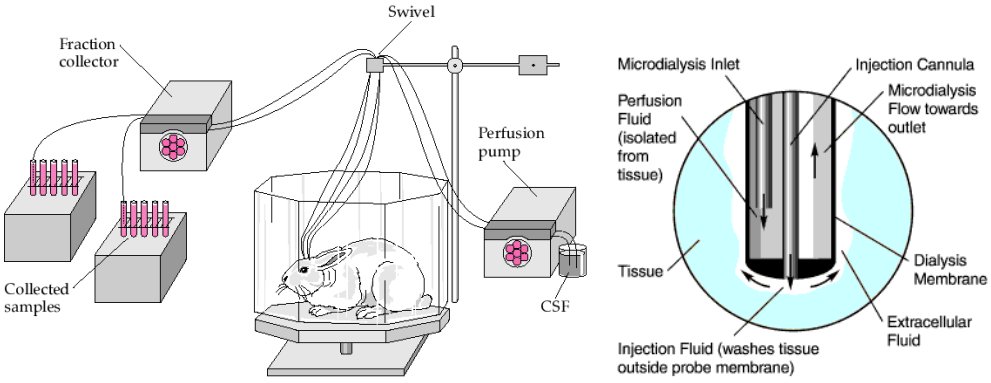
Neurochemical measurements are important in researches relating to cerebral ischemia since they are always involved in the neuronal injury and recovery mechanisms. Several methods are developed to measure concentration of neurotransmitter in order to investigate the mechanism of cerebral ischemia and evaluate the efficacy of treatments. These methods include postmortem tissue analysis and microdialysis. Microdialysis is preferred to be used for comparison with postmortem tissue analysis due to limited usefulness of tissue analysis. Postmortem tissue analysis could not estimate the change in the release rate of the transmitters. Besides, it cannot be used in behavioral studies or drug evaluation

since it is not sensitive enough to indicate for the small neurochemical changes (Ungerstedt 1991). Since postmortem tissue analysis is gradually being unpopular for stroke studies, only microdialysis will be discussed in the following.

2.3.4.1 Principle of Microdialysis

Microdialysis mimics the passive function of a capillary blood vessel by perfusing a thin dialysis tube which is implanted into the tissue. The composition of extracellular fluid (ECF) is reflected by the concentration of compounds in the perfusate due to the diffusion of substances back and forth over the dialysis membrane. This technique can be carried out in the intact tissue of the living, awake and freely moving animals, with minimal damage to blood-brain-barrier (BBB) and the experiment could continue for hours or days. The principle is that a tubular dialysis membrane is introduced into the tissue or placed into contact with a moist surface, then the tube is perfused with a liquid (perfusate) which equilibrates with the fluid outside the tube by diffusion in both directions. The composition of perfusate should be as close as possible to normal physiological levels for the most essential substances in ECF environment. The flow of the perfusate is kept as low as possible, approximately 0.1-5μl/min, in order to remove as little as possible and thus to minimize the interference with normal physiology. Microdialysis set-up includes probes, syringe pump, microfraction collector for collecting samples, injector for on line injections into a chromatograph for analysis, and syringe selector which could avoid introduction of air bubbles into the perfusate when changing the perfusion liquid (Ungerstedt 1991). The microdialysis samples are analyzed by high

performance liquid chromatography (HPLC), for example lactate, pyruvate and ascorbate can be detected by UV light at wavelength 214nm whereas, adenosine, inosine and hypoxanthine can be detected by UV light at 254nm (Hillered and Persson 1991). Figure 2.17 demonstrates the microdialysis set-up for measuring chemical changes in an awake rabbit.



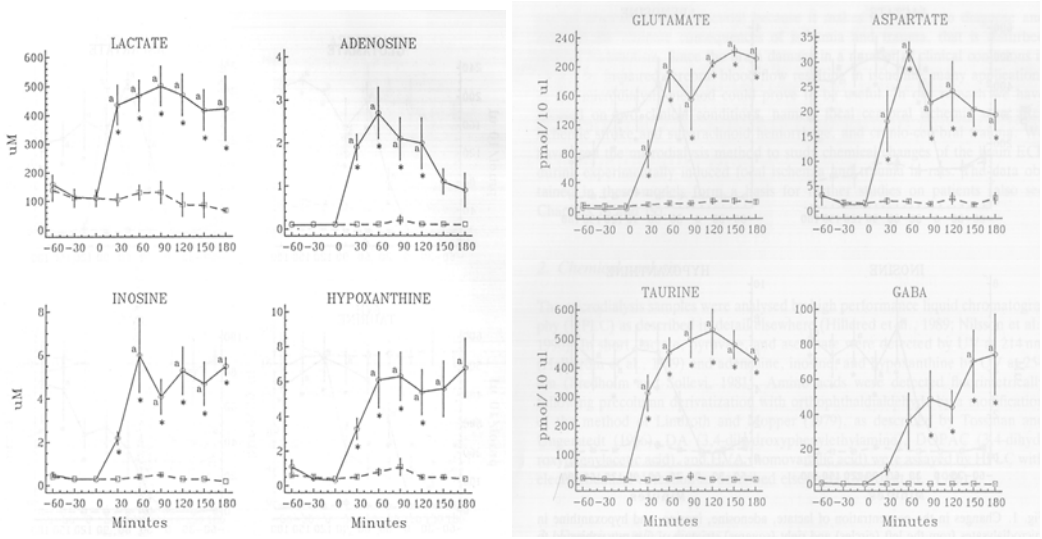
(a) (b)

Figure 2.17 (a) Brain sampling devices such as the push-pull cannula or microdialysis probe allow the collection of samples in a freely moving animal under relatively normal conditions (Emory University 2004); Microdialysis probe: Perfusing liquid enters the proximal end of injection cannula and flows distally all the way to its end where it changes direction and returns in the space between the cannula and the membrane where the microdialysis takes place (BAS Analytics 2004)

2.3.4.2 Microdialysis for cerebral ischemia

Intracerebral microdialysis allows measurement of important chemical changes of extracellular fluid (ECF) in brain in the course of cerebral ischemia. It can be done by implanting microdialysis probes stereotaxically into the lateral portion of caudate-putamen, which is the common location of infarcts, bilaterally two hours before middle cerebral artery occlusion procedures. The extracranial ends of the probes were fixed to the skull bone with dental cement. The systems were perfused with Ringer solution at a flow rate of 2µl/min and dialysates were sampled in three

30-min fractions before and after MCAo to allow analysis of a number of metabolites. MCAo causes dramatic increase in the ECF level of energy related metabolites including lactate, adenosine, inosine and hypoxanthine, which indicates a profound disturbance in energy metabolism caused by reduction of regional cerebral blood flow. Additionally, transmitters such as glutamate, aspartate, taurine and GABA also increase in a large extent. All these chemical changes during ischemia can be measured by microdialysis (Figure 2.18) (Hillered and Persson 1991; Benveniste and Hansen 1991).



(a) (b)

Figure 2.18 (a) Changes in concentrations of lactate, adenosine, inosine and hypozanthine in microdialysates from the left (circles) and right (square) striatum of five rats subjected to focal ischemia on the left side at time 0 min; (b) Changes in concentrations of glutamate, aspartate, taurine and GABA in microdialysates from the left (circles) and right (square) striatum of four rats subjected to focal ischemia on the left side at time 0 min (Benveniste and Hansen 1991)

2.4 Stroke Rehabilitation

Early after stroke, spontaneous recovery occurs rapidly and substantial improvement can be noted in motor function. It then proceeds at a slower pace until a plateau in

motor function is reached (Page *et al* 2004). Recovery of normal motor function might be possible in mild deficits caused by small infarcts. In case of more severe motor deficits, spontaneous recovery might not be enough to restore the motor functions (Stein 2004). Therefore, post-stroke rehabilitation is still important to facilitate the spontaneous recovery and to reduce the handicapping effects caused by stroke (Goldstein 2001; Schaechter 2004). An effective rehabilitation strategy might promote early motor recovery and thus allows the people after stroke to reintegrate into the society. In the past, conventional stroke rehabilitation, which focused on compensatory approaches for motor impairments, was believed to have a little impact on the spontaneous neurologic recovery (Stein 2004). However, it has become clear, in recent years, that a variety of interventions have the potential to positively influence the motor recovery. Common clinical approaches for triggering motor recovery after stroke are constraint-induced movement therapy, robot-aided rehabilitation, virtual reality training, treadmill training, electromyography-biofeeback, functional electrical stimulation and acupuncture (Stein 2004).

2.4.1 Possible mechanisms of motor recovery after stroke

Development of new effective therapeutic strategies for people after stroke relies on a better understanding of the mechanism underlying recovery of function. Recent studies have proposed several theoretical mechanisms to explain the recovery of motor function after brain injury based on the results from brain imaging techniques, animal experiments, *in vivo* and *in vitro* experiments and clinical studies.

One concept is that the functions lost through damage are taken over, or learnt by the unoccupied or unassigned regions of the brain. Another mechanism is based on redundancy, which means that the functions are variously represented throughout the nervous system (Held 1993). Lashley suggested that particular function was mediated by all the tissues in a particular region. If part of the region was damaged, the remaining intact tissue could still continue to mediate that function. Thus, the effect of lesion would depend on the amount of tissue spared (Carr and Shepherd 1987). One widely accepted mechanism of recovery is based on the plastic nature of the brain, which allows anatomical rearrangements and physiological readjustments to occur after injury. It might be in the form of regenerative or collateral sprouting, resolution of diaschisis and denervation supersensitvity (Held 1993). Regenerative sprouting refers to new growth in damaged neurons whereas collateral sprouting is the new growth in undamaged neurons adjacent to the destroyed neural tissue. These two forms of neural sprouting would increase synaptic effectiveness and allow the new system to substitute for the destroyed synapses. Diaschisis theory, which was proposed by von Monakow, suggested that the widespread effects of processes such as edema and extracelluar blood flow, would cause a suppression of activity in areas far from the site of the lesion. Reversible changes may occur in undamaged synapses (Carr and Shepherd 1987). This accounts for temporary impairment of neural transmission and recovery of functions in early period following stroke. Denervation supersensitivity describes the increased sensitivity to remaining afferent input by an increased post-synaptic responsiveness to

neurotransmitter substances after the axons and terminals degenerate due to the ischemic injury (Carr and Shepherd 1987).

2.4.2 Motor recovery by treadmill exercise

The effects of treadmill exercise on motor recovery after stroke have been extensively investigated in both clinical and animal studies. Although this type of training is labor intensive and poses a significant practical challenge in clinical settings, it is still widely used in lower extremity training for people after stroke.

2.4.2.1 Clinical studies

Clinical studies have been carried out in people after stroke regarding the efficacy of exercise on rehabilitation. It was found that treadmill exercise could improve walking quality, cardiovascular fitness and muscle strength. In Ada and colleagues' study, the results showed gained and maintained improvements in walking speed, walking capacity and step length in the treadmill trained group. These would definitely provide a better ambulation after stroke (Ada *et al* 2003). In Macko *et al*'s study, treadmill training in hemiparetic subjects with chronic stroke could reduce energy expenditure and improve gross motor efficiency. Besides, aerobic exercise training produced significant reductions in both the steady state oxygen consumption and respiratory exchange ratio (Macko *et al* 2001; Macko *et al* 1997). Smith *et al* have shown progressive treadmill aerobic exercise training might be beneficial to people after stroke by gaining in strength and reduced reflexive torque/time production in their hamstring muscles (Smith *et al* 1999).

2.4.2.2 Animal studies

Clinical studies have many limitations and the variations from individuals are large. Animal studies allow researchers to reproduce stroke under a controlled conditions. Moreover, severity and location of stroke could be controlled. These could minimize the variations and facilitate researchers to conduct parallel studies to compare different treatment protocols (de Lecinana *et al* 2001; Hunter *et al* 1998).

Recent animal studies have shown the protective effect of treadmill exercise prior to stroke incidence by Wang *et al* (Wang *et al* 2001). It was found that the pre-training might reduce the infarction volume and edema caused by middle cerebral artery occlusion (Wang *et al* 2001). Endres and colleagues had conducted similar study and has shown that regular physical activity could reduce cerebral infarct size and functional deficits by upregulating eNOS expression, increasing NO-dependent vasodilation and augmenting cerebral blood flow levels (Wang *et al* 2001). In Ang *et al*'s study, it was revealed that exercise promote the number of cholinergic neurons, which expressed the nerve growth factor (NGF). Besides, the study also provided results to support the efficacy of exercise in the reduction of infarct volume (Ang *et al* 2003). Similarly, there are animal studies showing benefits of exercise after stroke. Yang *et al* had shown the benefits of early training after stroke (24 hours after stroke) using cerebral ischemia model in rats. The rats were prescribed 1-week treadmill training, 5 days per week and 30 minutes per day. It was found that in the brain infarction volume was reduced in the rats (with 10 rats per group) with ischemic stroke (Yang *et al* 2003a). Other studies showed similar results (Yang *et al* 2003b; Marin *et al* 2003), as shown in Figure 2.19. In a study

conducted by Lee *et al*, it was noted that exercise suppressed the apoptosis in dentate gyrus (Lee *et al* 2003). However, Risedal *et al* found that there was loss of cortical tissue in infracted hemisphere in training group and it was explained by the release of glutamate which was triggered by motor activity (Risedal *et al* 1999; Meeusen *et al* 2001). High concentration of glutamate would kill neurons and trigger other damaging mechanisms and thus leading to more severe brain injury (Risedal *et al* 1999). In fact, effect of exercise has long been addressed regarding whether it is harmful or beneficial to ischemic brain. The conflicting results might be due to different intensities of interventions prescribed in the studies as shown in Table 2.4. However, there is lack of knowledge regarding the correlation between the intensity of exercise and the extent of brain damage, or whether the release of neurotransmitters triggered by exercise is graded.

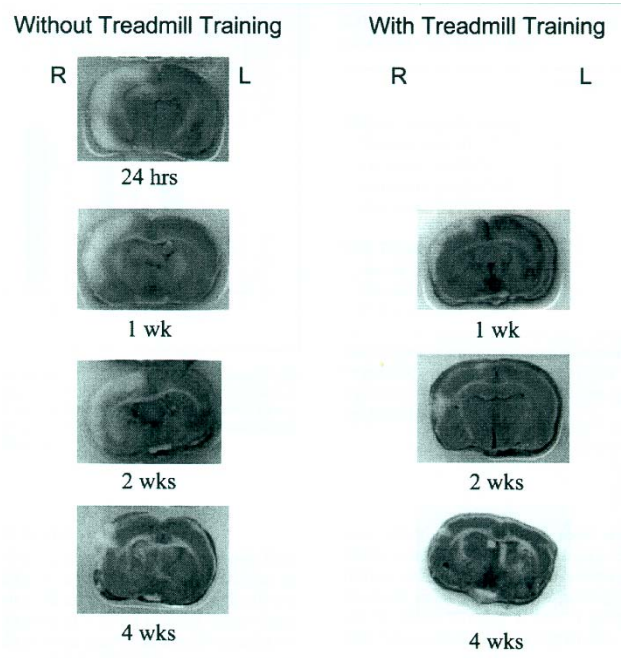


Figure 2.19 TTC stained brain slices from each condition after middle cerebral artery occlusion in rats. White area indicates infarct area whereas deep grey area indicates unaffected (normal) area. Infarct volumes of rats without treadmill training (left) were larger than that with treadmill training (right) (Yang *et al* 2003)

Table 2.4 Recent researches investigating the effect of exercise using animal models with cerebral ischemia (Endres *et al* 2003; Wang *et al* 2001; Ang *et al* 2003; Yang *et al* 2003a; Yang *et al* 2003b; Marin *et al* 2003; Lee *et al* 2003; Risedal *et al* 1999). *Note: ~ values were estimated from the graphs. *p<0.05; **p<0.001. Stained slice commonly included the measurement of direct and indirect infarct volume, and edema index.*

Ref	Species	Method to induce stroke	Interventions	Measurements	Mean (Control vs Ex)	Findings
Endres *et al* 2003	129/SV wild-type mice	Intra-luminal	Prior to stroke; Treadmill 5days/wk, 3wks, 12m/min, 30min/day	Neurological scores (Bederson)	Score 0: 0% vs 25%	Physical activity decreased stroke risk by increasing blood flow and reducing brain injury during cerebral ischemia
				Stained slice	* ~110 vs 75 mm^3	
				eNOS expression	* Ex: ~380% control	
Wang *et al* 2001	SD rats	Direct	Prior to stroke; Treadmill 5days/wk, 2wks, 20m/min, 30min/day	Stained slice	**~15% vs 6%	Treadmill training reduced ischemic brain damage after stroke
Ang *et al* 2003	Wistar rats	Direct	Prior to stroke; Treadmill, 30m/min; 50min/day, 4-12wks	Stained slice	*~ 175 vs 125 mm^3	Physical exercise increased nerve growth factor and facilitate proliferation of its receptive cholinergic neurons
				NGF expression	*~ 0.055 vs 0.085	
				p75 expression	*~0.8 vs 1.4	
Yang *et al* 2003a	SD rats	Direct	After stroke; Treadmill, 5days/wk, 2wks, 20m/min, 30min/day	Stained slice	*143.6±15 vs 102.6±6.5 mm^3	Reduced infarct volume in early training group
				Median of neurological scores (Menzies)	**2 vs 0	
Yang *et al* 2003b	SD rats	Direct	After stroke; Treadmill, 5days/wk, 1-4wks, 20m/min, 30min/day	Stained slice	1wk: *~140 vs 100 mm^3 2wks: *~140 vs 80 mm^3 4wks: **~95 vs 25mm^3	Treadmill training after focal cerebral ischemia significantly improves neurological outcome
				Median of neurological scores (Menzies)	1wk: *2 vs 0 2wks: *2 vs 0 4wks: 0 vs 0	
Lee *et al* 2003	Mongolian gerbils	Transient global ischemia	After stroke; Treadmill, 7days, 8m/min, 30min/day	TUNEL staining	* 76±2.87 vs 44±1.15m^2	Treadmill exercise may protect cells from apoptotic death and aid in recovery after stroke
				Caspase-3 IHC	*~90±7.64 vs 36±6.48 mm^2	
				BrdU IHC	*~145±11.72 vs 96±11.2 mm^2	
Risedal et al 1999	SHR	Direct	After stroke; 5days/wk, 4 weeks, Rotating pole, hanging on rope, keep balance on inclined plane, 1hr/day	Stained slice (cortex + thalamus)	*~45 vs 70%	Cortical infarct volume was larger in early training group than late training and control groups
				Rotarod	*~1.5 vs 4 min (10turns)	
				Limb placing (median)	5 vs 11	
				Water maze (Day 1)	Time: *~250 vs 225 sec Distance: *~70 vs 60 m	

In a study using healthy rats, it was found that brain plasticity could be induced by exercise. Studies have shown that exercise activated molecular and cellular cascades, which supported and maintained brain plasticity (Farmer *et al* 2004; Cotman and Berchtold 2002). It also induced expression of genes associated with plasticity, for example, genes encoding brain-derived growth factors (BDNF) in hippocampus (Farmer *et al* 2004). BDNF supports survival and growth of many neuronal subtypes and acts as a key mediator of synaptic efficacy, neuronal connectivity and use-dependent plasticity. Besides, exercise promoted neurogenesis and functional changes in neuronal structure in brain (Cotman and Berchtold 2002). Histological evaluation with in situ hybridization showed that nerve growth factors (NGF) mRNA level in the dentate gyrus and area CA4 of the hippocampus was increased significantly, following 7 nights with exercise prescribed to healthy rats (Neeper *et al* 1996). NGF stimulates neurite growth, direct incoming growth cones and promote survival of neurons (Matthews 2001). However, there was limited information provided in literatures regarding the exercise-induced plasticity in stroke model of rats (Kleim et al 2003).

2.4.3 Motor recovery by neuromuscular electrical stimulation

Treadmill exercise could affect the functioning and recovery of damaged brain and it might promote brain plasticity. It is still not clear that neuromuscular electrical stimulation (NMES), in which bursts of short electrical pulses was used to generate repetitive active muscle contraction (Merletti *et al* 1979), could trigger similar effects of treadmill exercise. Electrical simulation was believed to affect both peripheral systems and the central nervous systems. In the peripheral system,

electrical stimulation on muscles could reverse muscle atrophy, alter the muscle characteristics, modify spasticity and rigidity, accelerate peripheral nerve regeneration and improve blood circulation at the stimulated areas (Daly *et al* 1996). In central nervous system, active repetitive movement might facilitate functional reorganization in hemiplegia, by modifying the excitability of specific motor neurons (Chae and Yu 1999). A mechanism of the therapeutic effects of neuromuscular electrical stimulation suggested by Chae *et al* was that a cyclic electrical stimulation on muscles would produce an active repetitive movement. These movements would either trigger one or all of the following pathways – functional reorganization where intact areas take up the function of damaged area, or activation of alternate descending pathways and structures on lesioned side, or activation of pathways and structures in contralateral undamaged hemisphere. These then facilitated motor relearning and thus improve voluntary movement (Chae and Yu 1999). Another mechanism suggested by Dobkin *et al* was that electrical stimulation directly activate cutaneous and muscle-tendon afferents and the mechanical events of muscle contraction would have inputs to the spinal cord and thus to the brain. The restoration of motor functions by long-term NMES was believed to be due to formation of novel neural circuits, which was a result of increased release of neurotransmitters, increased in number of receptors and sprouting axons (Park *et al* 2004).

2.4.3.1 Clinical studies

Liberson *et al* pioneered to use electrical stimulation to improve the gait of hemiplegic subjects. In some cases, a specific recovery of voluntary power in

paralyzed movement may be observed (Rushton 2003). Glanz *et al* conducted a meta-analysis on post-stroke rehabilitation advocated that FES can promote recovery of muscle strength (Glanz *et al* 1996). In a review by Popovic *et al*, it was noted that electrical stimulation treatment could give better results in retraining grasping and walking functions in people with spinal cord injury (Popovic *et al* 2001). Other studies showed that neuromuscular electrical stimulation improved voluntary ankle dorsiflexion and knee flexion (Merletti *et al* 1979; Cozean *et al* 1988), control of weight acceptance onto a paretic limb (Waters *et al* 1988) and increased walking speed (Bogataj *et al* 1997) in people after stroke. Advanced techniques for brain imaging and analyzing electromagnetic properties of brain such as functional magnetic resonance imaging (fMRI) and transcranial magnetic stimulation (TMS) revealed the altered post-stroke activation patterns of specific brain structures, which might be an implication of plastic reorganization (Rossini and Pauri 2000). Brain activation in specific neural regions was observed using fMRI techniques, when applying neuromuscular electrical stimulation on lower limb in healthy adult subjects (Smith *et al* 2003). Han *et al* revealed in his study that significant activation on contralateral primary sensorimotor cortex (using fMRI) by neuromuscular electrical stimulation on the wrist extensor muscles was observed in healthy subjects, as shown in the fMRI pictures in Figure 2.20 (Han *et al* 2003). Kimberley *et al* found that repetitive movements with NMES were effective in producing improvements in hand function and were associated with an increase in the cortical intensity in the ipsilateral primary sensory cortex. No activation in

motor cortex might imply that there was no active engagement during the rehabilitation to facilitate motor cortex changes (Kimberley *et al* 2004).

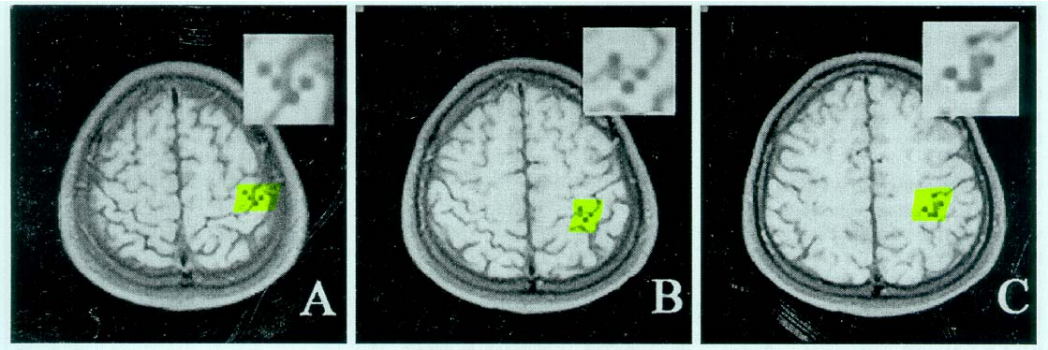


Figure 2.20 The contralateral primary motor cortex and primary sensory cortex activation maps of neuromuscular electrical stimulation on the right wrist extensor muscles. The inset images in the right upper corners are magnified images of the region including the activated pixels (Han *et al* 2003).

2.4.3.2 Animal studies

Animal studies have been conducted to investigate the effects of electrical stimulation on peripheral systems, usually muscles, using rats with atrophied muscles and hemispinalized cats. Salter *et al* had demonstrated the effect of neuromuscular electrical stimulation on the atrophied muscles, in which the atrophy was induced by blocking the sodium channels of muscle nerves using tetrodotoxin (TTX) in rats. It was found that at long-term program of low-frequency stimulation (2 pulse per second, using implanted microstimulator), the average level of atrophy by measurement of soleus and tibialis anterior of rats were decreased. The result suggested that NMES might provide significant protection from muscle atrophy (Salter *et al* 2003). In Kernell *et al*'s study, the effects of stimulating muscles with 10Hz and 100Hz trains of 10 pulses delivered every few seconds continuously for four and eight weeks. This intervention, carried out in hemispinalized cat, led to fibre transformation toward type I profiles with small diameters and a reduction of

muscle capability to produce titanic tension (Kernell *et al* 1987). Apart from changing the muscles properties, the effects of neuronmuscular stimulation on impaired central nervous system and damaged brain have not been well studied.

In vivo effects of neuromuscular electrical stimulation on brain after cerebral ischemia have not been reveals previously. Moreover, it is currently unclear whether neuromuscular electrical stimulation triggers the similar effect on the brain after cerebral ischemia which is induced by treadmill exercise. In addition, neurochemical effects of the two interventions on the brain after stroke are not well studied.

CHAPTER 3 METHODOLOGY

The experiment comprised three parts – a pilot study, a study based on assessment scores and stained brain slices (Study I) and a microdialysis study (Study II). Figure 3.1 showed the outline of latter two parts of the experiment. Study I aimed at investigating the effects of treadmill exercise and neuromuscular electrical stimulation (NMES) on neurological and histological outcomes after stroke. Study II further looked into the events taken place inside the brain after stroke while the treatments were prescribed, in terms of the levels of certain neurotransmitters released in the hippcomapus. Levels of aspartate, glutamate, taurine and gamma-aminobutyric acid (GABA) were measured using in vivo microdialysis technique, for their important roles in cerebral ischemia (refer to Section 2.1.4).

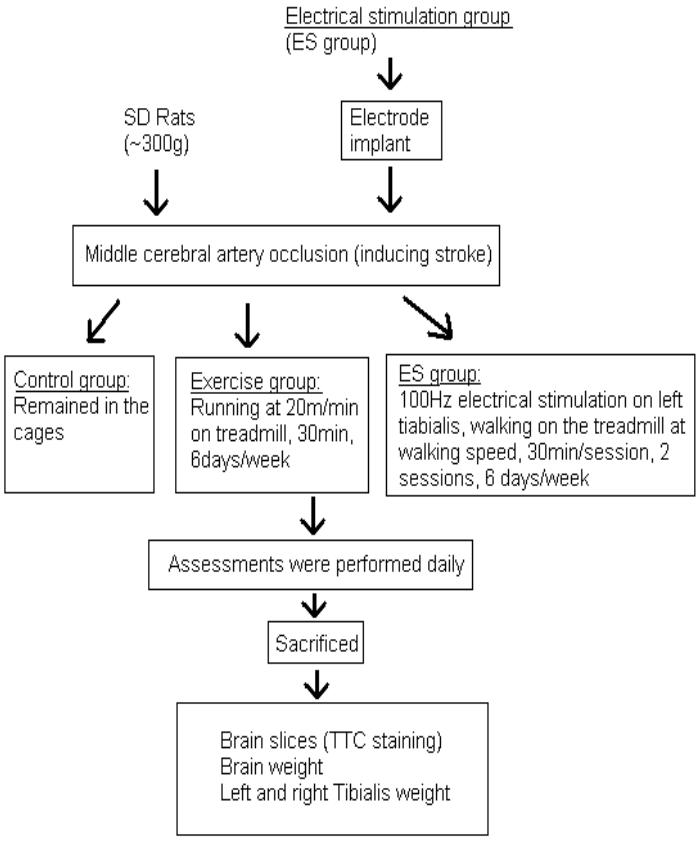


Figure 3.1 (a) Outline of the experiments with evaluations of the effects based on assessment scores and stained brain slices

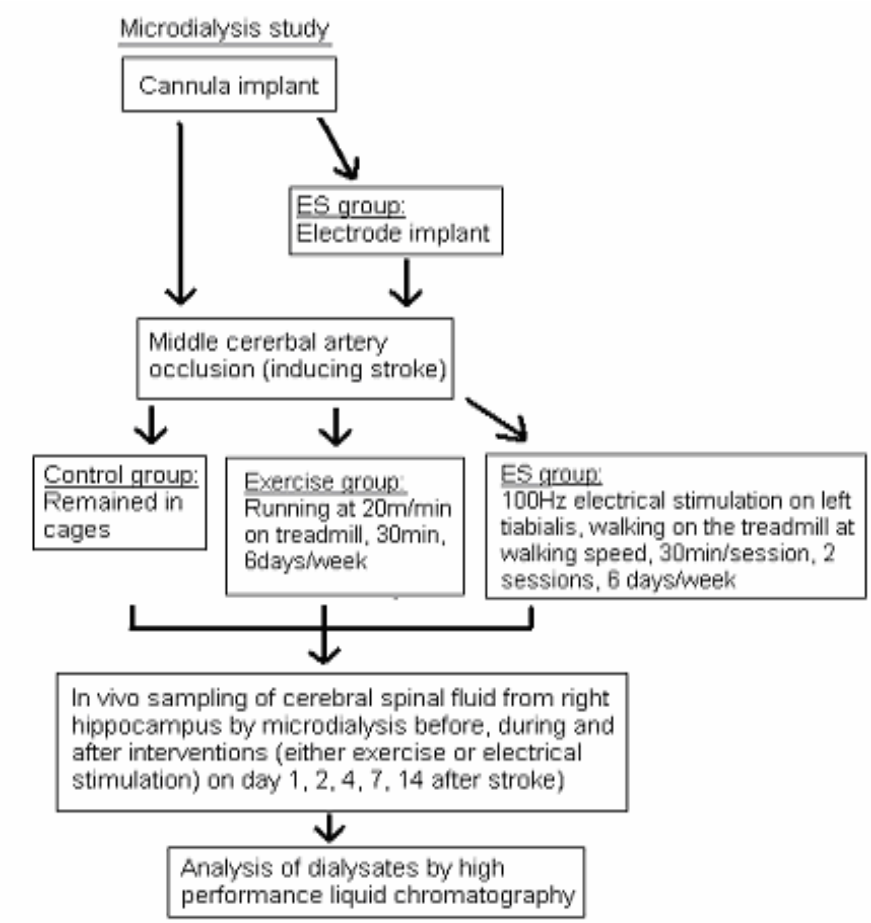


Figure 3.1 (b) Outline of the experiments evaluation based on concentration of neurotransmitters by microdialysis

3.1 Ethical consideration

The Animal Ethics Review Committee of the Hong Kong Polytechnic University reviewed and approved the procedures of this study (Appendix I). The handling and husbandry of the animals were in strict compliance with their recommendations.

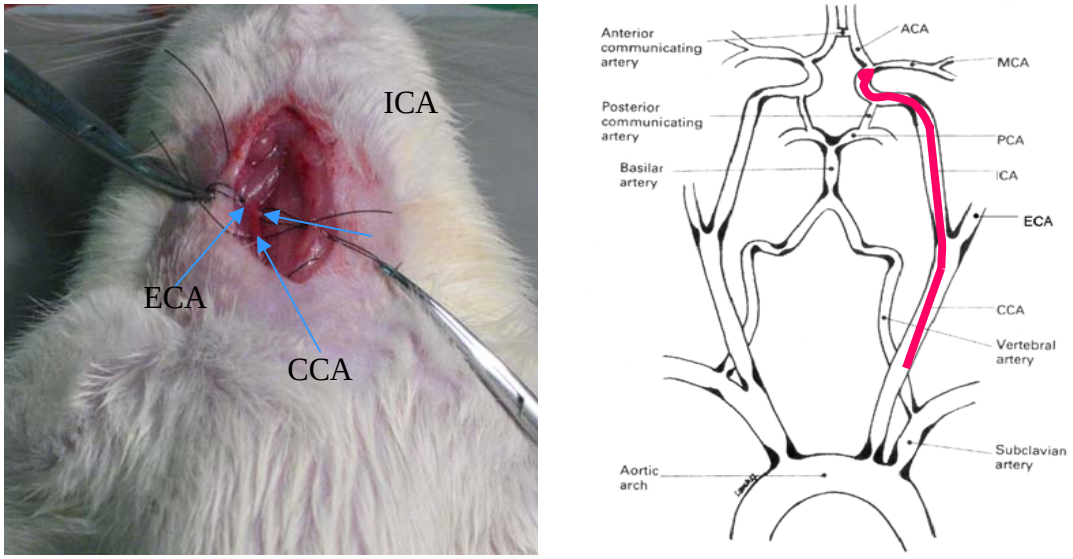
3.2 Pilot study

A pilot study was carried out to determine the suitable duration to occlude the artery, which could produce considerable infarction with survival time over two weeks. Intraluminal suture technique was chosen as a method to induce cerebral ischemia in rats as it produced focal infarction while no craniotomy was required, and its close resemblance to clinical stroke (refer to Table 2.1 in Chapter 2). 25 young male Sprague-Dawley rats were used in the pilot study. All rats were provided by the Central Animal Facilities of the Hong Kong Polytechnic University. Middle cerebral artery occlusion (MCAo) was carried out to induce stroke in all rats with different durations of occlusions: 40 minutes (n=9), 60 minutes (n=7), 75 minutes (n = 4), 90 minutes (n=2) and 120 minutes (n=3). All rats were allowed to receive water and food *ad libitum*. All rats were sacrificed two weeks after MCAo procedures by overdose anesthesia. Brain slices were obtained and stained using 2,3,5-triphenyltetrazolium chloride.

3.2.1 Middle Cerebral Artery Occlusion

Focal cerebral ischemia was induced using the middle cerebral artery occlusion (MCAo) model in all rats. Anesthesia was induced with Ketamine and Xylazine (40 mg/kg Ketamine, 5 mg/kg Xylazine, Intraperitoneal). The right middle cerebral artery (MCA) was occluded by the intraluminal suture technique as described by Longa *et al* with some modifications (Longa et al 1989). The right carotid region was exposed through a midline cervical incision and the external carotid and common carotid arteries were ligated. A 3-0 monofilament nylon suture, whose tip

rounded by heating and coated with poly-l-lysine, was introduced from the carotid bifurcation into the internal carotid artery until a mild resistance was felt, thereby occluded the origin of the MCA. Re-circulation was established by gentle withdrawal of the suture. During surgery, temperature was maintained in the normal range by a heating lamp. After surgery, Enrofloxacin was injected intramuscularly for prophylaxis. The painkiller, Buprenorphine (0.1 – 0.3 mg/kg) was prescribed immediately after surgery, then once more one day after. And if necessary, the third dose was prescribed on the third day.



(a) (b)

Figure 3.2 (a) Positions of common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA). CCA and ECA were ligated whereas ICA was not ligated. Instead, ICA would be temporarily clamped with a micro aneurysm clip. (b) Diagram showing the insertion of intraluminal suture via CCA to middle cerebral artery (MCA)

After the incidence of stroke, the rats usually became 'dispirited', the hair became fluffy and lost its luster. Paralyses usually appeared in limbs, which are contralateral to the damaged hemisphere. Moreover, the shoulder on the paretic side might show

adduction and the muscle strength of the affected forelimb was weakened (Inoue et al 2002).

3.2.2 Brain Infarction Volume

2,3,5-Triphenyltetrazolium chloride (TTC) staining was used to quantify the brain damage, for its specificity to ischemic tissues (refer to Section 2.3.3.1). Rats in all groups were sacrificed two weeks after MCAo procedures by overdose anesthesia. The brain was immediately removed from the cranium and dissected into 2mm sections using brain matrix (Figure 3.3a). The brain slices was immersed in 2% solution of 2,3,5-TTC in normal saline at 37 ^{o}C for 30 min. They were then fixed in 10% formaldehyde. Cross-sectional area of infarction of each slice was measured by image analyzer software (UTHSCSA Image Tool for Windows version 3.00). Brain infarction volume was obtained by summation of the infarct volume of all slices. Figure 3.3b shows the brain slices used for measuring the brain infarction volume.

(a) (b)

Figure 3.3 (a) Brain matrix; (b) Brain slices taken from cerebral ischemic rat. The white area indicates the infarction due to middle cerebral artery occlusion

3.3 Study I: Evaluations of the effects of interventions based on assessment scores and stained brain slices

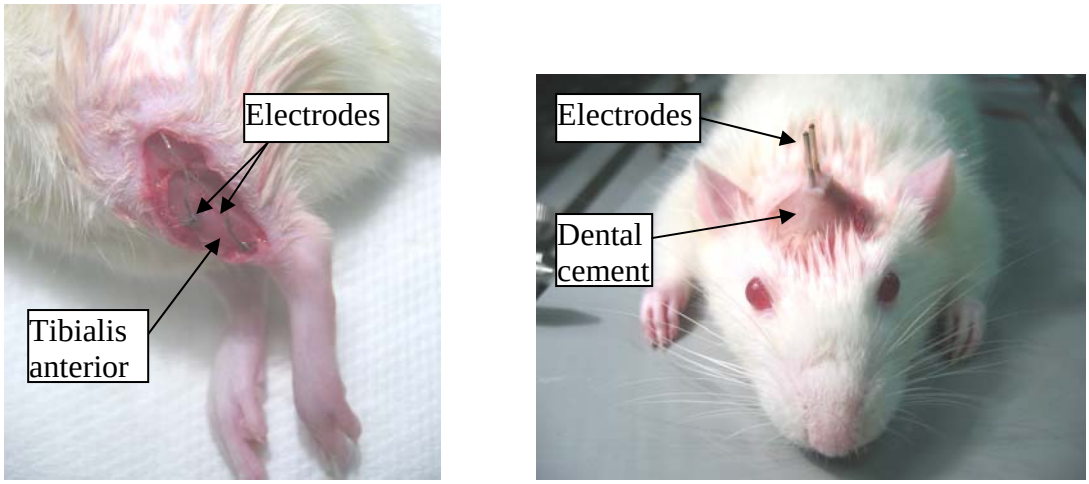
151 young male Sprague-Dawley rats were used in Study I. Rats were provided by the Central Animal Facilities of the Hong Kong Polytechnic University and the Laboratory Animal Services Centre of the Chinese University of Hong Kong. Only 55 rats were included in the study whereas others were either dead or failed to induce stroke after MCAo. Rats were randomly assigned into four groups, as described in the following. Middle cerebral artery occlusion (MCAo) was carried out to induce stroke in all rats as described in Section 3.2.1, except those in the sham-operation group. For the rats in the sham-operation group, the surgical procedures were the same as described, but intraluminal filament was not inserted into the artery and therefore, no cerebral ischemia was induced. All rats were allowed to receive water and food *ad libitum*.

1. Sham-operation group (SHAM group) – Surgery of MCAo was done without inserting the filament, thus, no cerebral ischemia was induced (n=6)
2. Control group (Control group) – MCAo only (n=17)
3. Exercise group (EX group) – Treadmill training after MCAo induction (n=17)
4. Electrical stimulation group (NMES group) – Electrical stimulation on hind limb muscle after MCAo induction (n=15)

3.3.1 Implantation of electrodes

Rats in NMES group were anaesthetized with Ketamine and Xylazine (40 mg/kg Ketamine, 5 mg/kg Xylazine, Intraperitoneal). Hair on the left hindlimb was

removed and tibialis anterior (TA) muscle was exposed. Two Teflon-coated stainless steel wires (AW633, Cooner Wire, US) were passed subcutaneously from the incision on the limb to the exposed skull. Electrodes were made by stripping insulation off the end of the wires and looping them around the belly of TA muscle (Dow et al 2004). The two electrodes were placed at the 1/3 of the total length of TA muscle, from distal and proximal ends respectively. Another ends of the two wires were fixed on the skull with stainless steel screws and dental cement (Marqueste et al 2002). After surgery, Enrofloxacin was injected intramuscularly for prophylaxis. The painkiller, Buprenorphine (0.1 – 0.3 mg/kg) was prescribed immediately after surgery. The rats were allowed to recover for 1 week before inducing stroke by middle cerebral artery occlusion.



(a) (b)

Figure 3.4 (a) Electrodes attached at the TA muscle; (b) Another ends of electrodes were fixed with dental cement on the skull.

3.3.2 Protocols of interventions

Rats in SHAM and Control groups remained inactive in the cage and they were sacrificed two weeks after sham operation (SHAM group) and induction of cerebral ischemia (Control group) respectively.

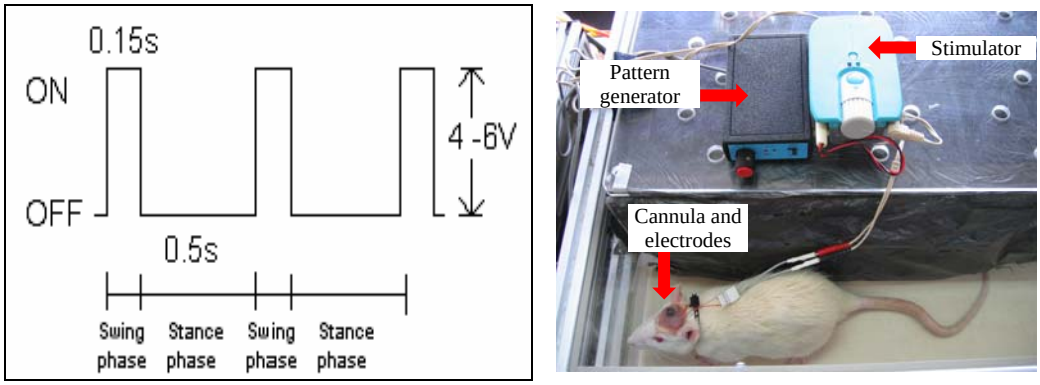
3.3.2.1 Treadmill training

EX group was prescribed treadmill exercise after cerebral ischemia induction. They were scheduled to start a two-week treadmill training 24 hours after stroke incidence and were sacrificed immediately after completing the training. The treadmill training was 30 min per day, 5 days a week with speed 20 m/min and with zero degree of inclination (Yang et al 2003a; Yang et al 2003b).

3.3.2.2 Neuromuscular electrical stimulation

Animals from NMES group were stimulated for two consecutive weeks (twice a day, 30 minutes/section, five days/week). They were stimulated with a biphasic stimulation (4-6V). Frequency of the stimulation was 100Hz (Michel et al 1996) and the pulse width was set to 300 μs. The stimulation was delivered by a clinical stimulator. Figure 3.5a shows the stimulation pulse and pattern, which was generated by the pattern generator. The stimulation pattern was based on the findings regarding the rat gait by Gillis and Biewener (Gillis and Biewener 2001; Appendix III). The rats with implanted electrodes were allowed to walk on the treadmill with speed 5m/min while electrical stimulation was applied on the TA muscle on the affected side (left) (Figure 3.5b). The treadmill was used to keep the

rats walking while applying the stimulation and it was assumed that the effect of walking at this slow speed was negligible on the parameters being measured. However, the stimulation pattern and the gait might not be synchronized in some rats since these rats sometimes did not walk on the running belt of the treadmill. There was at least 3 hours between the first and the second session, which aims at allowing the muscle to take rest. This is in accordance to the usual clinical practices (Dimitrijevic and Dimitrijevic 2002; Greve et al 1993; Francisco et al 1998; Mayr et al 2002).



(a) (b)

Figure 3.5 (a) Stimulation pattern generated by the pattern generator. (b) Rat with implanted cannula and electrodes ran on the treadmill while neuromuscular electrical stimulation was applying by the clinical stimulator and pattern generator.

3.3.3 Evaluations & Analyses

All rats after cerebral ischemia were tested daily in the same sequence of assessments as shown in Table 3.1 during light cycle. They were sacrificed two weeks after MCAo procedures by overdose anesthesia. Brain was removed and stained for measuring brain damage as described in Section 3.2.2.

Table 3.1 Summary of assessments and *in vivo* testing for the rats

Assessments/ Testing
1. Body weight
2. Postural reflex test
3. Limb placing test
4. Brain infarction volume

3.3.3.1 Body weight of rats

Rats were weighed using precision balance daily after cerebral ischemia. The body weights were recorded in the morning everyday.

3.3.3.2 Postural reflex test

Neurological assessment was performed in all rats using Longa's scoring scale (Longa et al 1989), which was corresponding to the intralumnial suture model used in this study (refer to Section 2.3.1.1). The neurological deficits were graded according to Table 3.2. Forelimb flexion was tested by holding the rats gently by the tail and suspending it 1 m above the floor. Focal cerebral ischemic rats usually flexed their forelimb, which was contralateral to injured hemisphere as shown in Figure 3.6, whereas normal rat extended both forelimbs towards the floor (Bederson et al 1986). After testing the forelimb flexion, the rat was allowed to move freely and circling might be observed.

Table 3.2 Longa's scoring scale

Score	Deficit(s)
0	No neurologic deficit
1	Failure to extend forepaw of the affected side fully
2	Circling to the affected side
3	Falling to the affected side
4	Did not walk spontaneously and had a depressed level of consciousness

Figure 3.6 Rat with focal cerebral ischemic flexed forelimb (circled) when holding the tail gently and suspending 1m above the ground

3.3.3.3 Limb placing

This assessment was originally designed by De Ryck *et al* (De Ryck et al 1989), to examine sensorimotor integration in forelimb placing responses to visual, tactile and proprioceptive stimuli after stroke, reflecting the recovery of sensorimotor neocortex (refer to Section 2.3.1.2). The assessments being used in this experiment was modified by Olhsson *et al* (Ohlsson and Johansson 1995).

Forelimb	
Each forepaw (affected and unaffected sides) was graded in six tests. During tests 1 through 4, the rat was held in a soft grip by the examiner.	
Test 1	Limb placing was tested by slowly lowering the rat toward a table. At about 10 cm above the table. Normal rats stretched and placed both forepaws on the table (Figure 3.6)
Test 2	With the rat's forelimbs touching the table edge, the head of the rat was moved 45° upward while the chin was supported to prevent the nose and the vibrissae from touching the table. A rat with focal brain lesion might lose contact with the table with the paw contralateral to the injured hemisphere. (Figure 3.7a)
Test 3	Forelimb placement of the rat when facing a table edge was observed. A normal rat placed both forepaws on the table top. (Figure 3.7b)
Test 4	Recorded forelimb and hindlimb placement when the lateral side of the rat's body was moved toward the table edge. (Figure 3.7c)
Test 5	The rat was placed on the table and gently pushed from behind toward the table edge. A normal rat griped on the edge, but an injured rat might drop the forelimb contralateral to the injured hemisphere. (Figure 3.7d)
Test 6	Same as test 5, but the rat was pushed laterally toward the table edge (Figure 3.7e)

Hindlimb
Repeated Test 4 (Figure 3.7f) and Test 6 (Figure 3.7e) and observed the hindlimb placing behaviors. For each body side, the maximum score from the tests used is 16.

Scores	
0	no placing
1	incomplete and/or delayed (>2 seconds) placing
2	immediate and correct placing

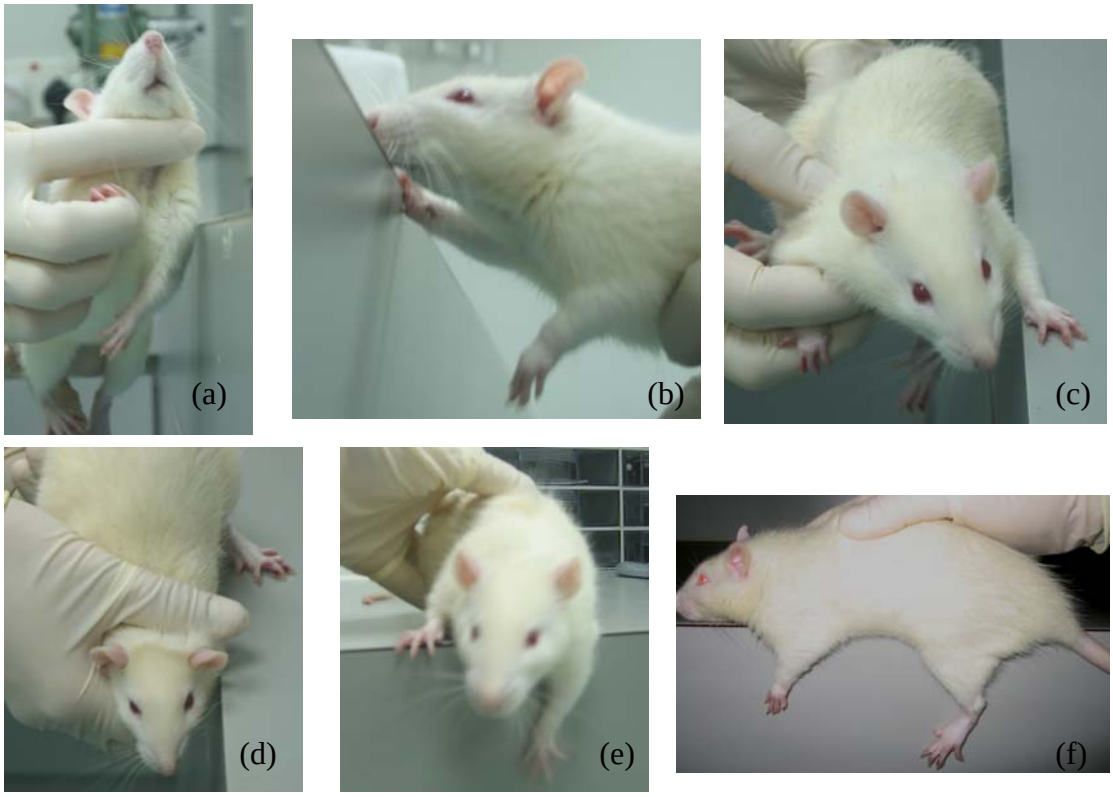


Figure 3.7 Limb placing test of rat 2 weeks after right focal cerebral ischemai. (a) Test 2 – forelimb on the affected side (left) did not placed on the table edge when the head was tilted. (b) Test 3 - Left forelimb did not placed on the table edge whereas right forelimb (unaffected side) placed on the table edge immediately when the rat faced the table edge. (c) Test 4 – Left forelimb placed on the table edge when the rat was placed near edge. (d) Test 4 (Hindlimb) – Left hindlimb placed on the table edge when the head was placed near the table edge. (e) Test 5 – Left forelimb did not placed on the table edge whereas right forelimb did the correct placing. (f) Test 6 (Forelimb & hindlimb) – Left forelimb and left hindlimb did not placed on the table edge when the rat was pushed laterally to the table edge.

3.4 Study II: Microdialysis study

In vivo intracerebral microdialysis was used to elucidate the hippocampal levels of neurotransmitters (glutamate, aspartate, taurine and GABA) in rats with and without treatments (Exercise or NMES) after stroke (refer to Section 2.3.4). 48 young male Sprague-Dawley rats were used in Study II. Rats were provided by the Animal Laboratory of Zhejiang University, China and the study was conducted at the department of Biomedical Engineering of Zhejiang University. Only 19 rats were

included in the study whereas others were either dead or failed to induce stroke after MCAo. Rats were randomly assigned into three groups – Control (n=6), EX group (n=8) and NMES group (n=5). All rats were allowed to receive water and food *ad libitum*. Guide cannula was implanted in all rats prior to the MCAo surgical procedures. In the NMES group, implantation of electrodes (as described in Section 3.3.1) was done together with the cannula implantation. The animals were allowed to recover from surgery for 1 week before inducing stroke. MCAo were induced in all rats as described in Section 3.2.1. Rats in Control group remained inactive in the cage. Treadmill exercise and neuromuscular electrical stimulation were prescribed to EX group and NMES group respectively 24 hours after stroke. The protocols of the interventions were in accordance to Section 3.3.2. All rats were sacrificed two weeks after induction of cerebral ischemia by overdoes anaesthesia.

3.4.1 Implantation of guide cannula

Animals were anaesthetized with chloral hydrate (0.4mg/kg, i.p.) and placed on stereotaxic frame. The skull was exposed and a guide cannula was implanted through a bore hole in the right hippocampus (AP: 5.8 mm; ML: 5.0 mm; DV: 3.0 mm) as described by Paxinos & Watson (Paxinos and Watson 1998), relative to bregma. The cannula was fixed with two screws and dental cement. In the NMES group, the wires were fixed together with the cannula. After surgery, Enrofloxacin was injected intramuscularly for prophylaxis. The painkiller, Buprenorphine (0.1 – 0.3 mg/kg) was prescribed immediately after surgery.

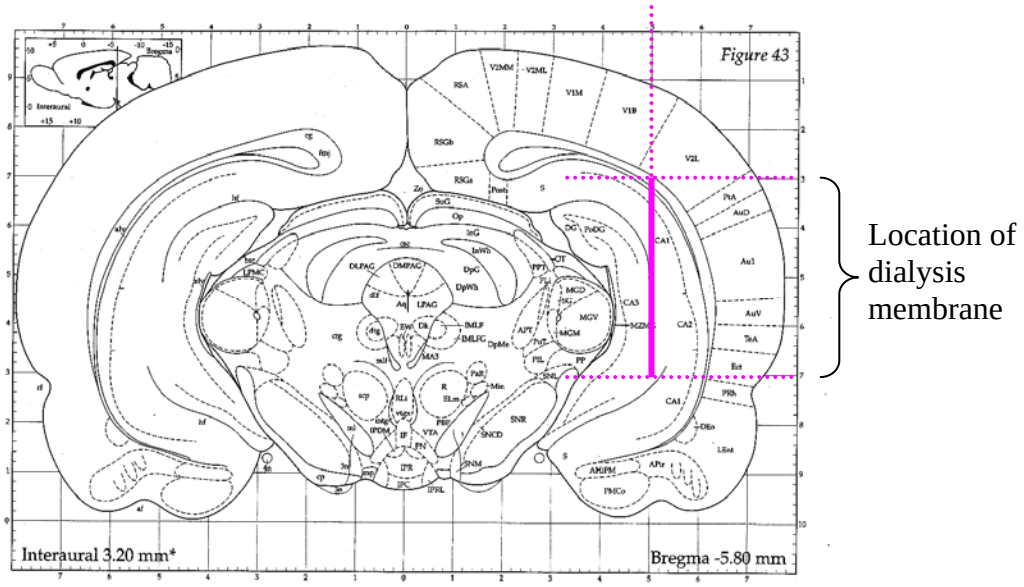
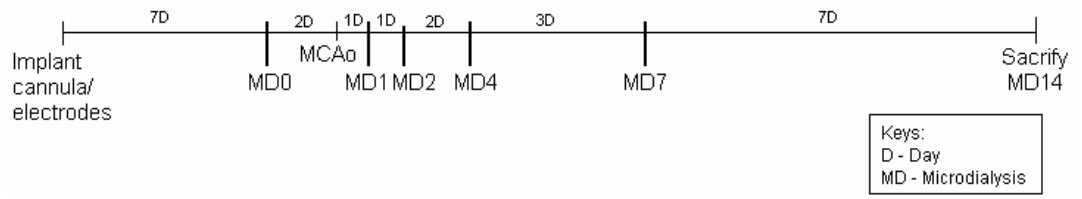


Figure 3.8 The dialysis membrane of the probe (solid line) would be located along the right hippocampus when the probe is inserted into the guide cannula. Neurotransmitters in the right hippocampus can be perfused through the membrane (Paxinos and Watson 1998)

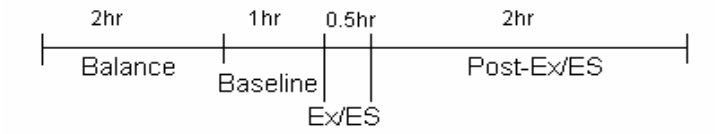
3.4.2 In vivo sampling using microdialysis

In vivo samplings using microdialysis were carried out at specified days (Before stroke, day 1, 2, 4, 7, 14 after stroke) as shown in Figure 3.9a. The microdialysis probe (BAS MD-2204, membrane length = 4mm) was inserted through the guide. The probes were connected to a microinfusion pump (BAS Inc.) perfused with artificial cerebrospinal fluid (mixed according to BAS Laboratory Manual of Microdialysis) at a constant flow rate of 2μL min^{-1}. As shown in Figure 3.9b, the sampling of dialysates began 2 hours after probe insertion. The microdialysis samples were collected every 15 minutes in vials. Samples were collected for 1 hour (4 samples) to verify stable basal conditions. The animals in EX and NMES group were then undergo 30-minutes treadmill exercise or electrical stimulation respectively (2 samples) (Figure 3.10). They were remained on the treadmill, which was switched off, for 2 hours after interventions (8 samples). The total collection

time was about 5.5 hours. The rats in Control group were remained on a non-moving treadmill for 5.5 hours and 14 dialysate samples were collected.



(a)



(b)

Figure 3.9 (a) Schedule of the surgeries and in vivo samplings using microdialysis. The *in vivo* sampling was carried out before stroke, on day 1, 2, 4, 7, 14 after stroke; (b) Time allocation of each rat for the in vivo sampling using microdialysis

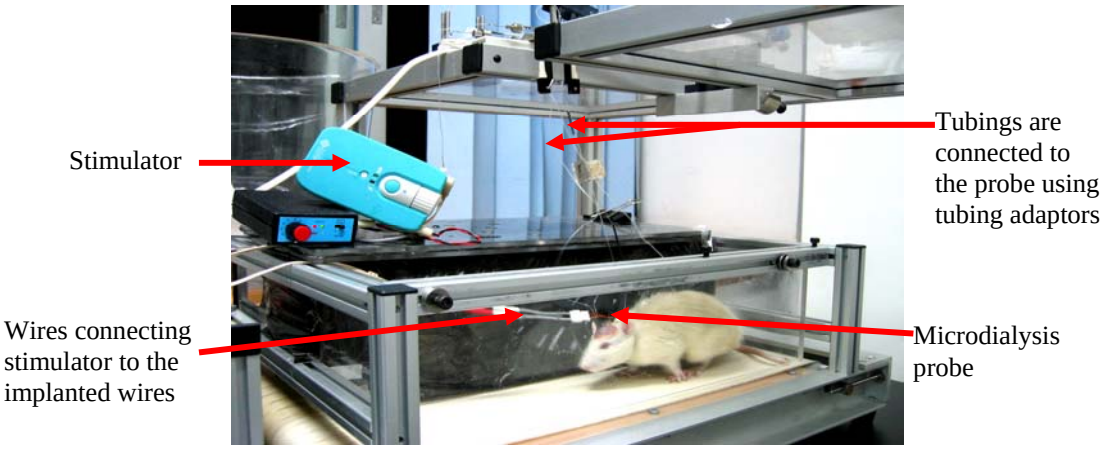


Figure 3.10 Rat running on treadmill with neuromuscular electrical stimulation, dialysates from the hippocampus were obtained every 15 minutes by microdialysis techniques.

Asparate, glutamate, taurine and gamma-aminobutyric acid (GABA) were assayed by High Performance Liquid Chromatography (HPLC) with fluorescence detection, using the precolumn derivatisation method with ortho-phthaldialdehyde and an

automatic system from Shimadzu Instruments consisting of a SCL-10A system controller, a RF10A XL Spectrofluorometric Detector, a CTO-10A column oven and two LC10AT VP pumps (Shimadzu, Kyoto, Japan). Excitation and emission wavelengths were set at 360 and 450 nm, respectively. 20μl microdialysate was used for derivatization with 10μl OPA reagent (Sigma-Aldrich, USA) diluted with 0.1M/L Boric acid buffer (pH 9.5) and 10μl Ethanolamine was added, which was used for normalization of the data. 20μl of this reaction mixture was injected directly into the HPLC system. The areas under the peaks of asparate, glutamate, taurine, GABA and ethanolamine were collected for further analysis.

3.5 Data Analysis

All statistical analyses were performed using SPSS version 13.0. A p-value of less than 0.05 was considered to be statistically significant.

3.5.1 Temporal analysis

Change of body weight of each rat at each day was calculated by subtracting the weight on that day by the weight before surgery of middle cerebral artery occlusion (weight before sham operation in Sham group). It was presented as mean change ± SEM for each group. It was hypothesized that there were differences in mean change of weight among groups at Day 0, 1, 2, 4, 7 and 14 and there were differences at Day 1, 2, 4, 7 and 14 compared with baseline (Day 0). Repeated two-way ANOVA followed by Bonferroni post-hoc analysis was used to test for significance. The neurological deficits scores and limb placing scores of

contralesional and ipsilesional sides (summation of scores from forelimb and hindlimb tests, maximum 16 on each side) of each day throughout the two weeks were presented as means ± SEM for each group. The hypotheses were that there were differences in scores among groups at Day 1, 2, 7 and 14 and there were differences at Day 2, 7, 14 compared with baseline. Data obtained from postural reflexes test were non-parametric. Statistical difference in neurological scores between groups was determined by Kruskal-Wallis test. Friedman test followed by Wilconxon signed ranks test was used to compare data within group at Day 1, 2, 7 and 14. Repeated two-way ANOVA followed by Bonferroni post-hoc analysis was used to test for significance in limb placing scores at Day 1, 2, 7 and 14.

In the microdialysis study, areas of the peaks of the amino acids obtained from HPLC were normalized by the area of ethanolamine peak (Figure 3.11). The concentration of each amino acid (in μM) was obtained by the calibration curve as shown in Figure 3.12. The calibration curve was obtained by assaying the four amino acids with known concentrations (0, 0.1, 0.2, 0.5, 1.0, 2.0, 2.5, 5.0, 10.0, 20.0 μM) using HPLC.

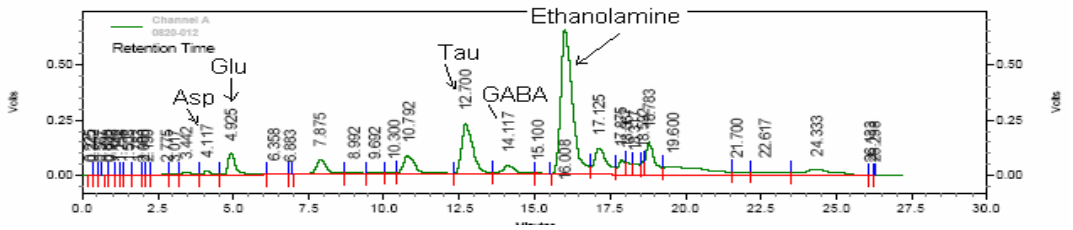


Figure 3.11 Graph obtained in HPLC showed the peaks of the amino acids and other chemicals inside the dialysates. From left to right: Aspartate (Asp), Glutamate (Glu), Taurine (Tau), GABA and Ethanolamine.

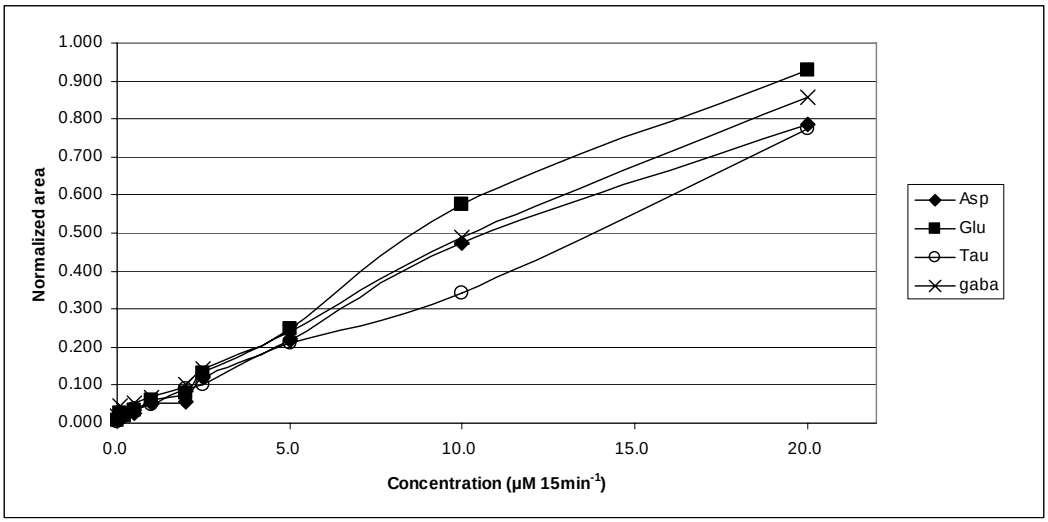


Figure 3.12 Standard curve for calculating the concentrations of aspartate, glutamate, taurine and GABA.

Equations obtained from trend lines of the curves (linear regression) shown in Figure 3.12 (trend lines are not shown) are as follows:

Aspartate: $y = 0.0401x + 0.013$, $R^2 = 0.9899$
Glutamate: $y = 0.0477x + 0.0134$, $R^2 = 0.9887$
Taurine: $y = 0.0375x + 0.0095$, $R^2 = 0.9956$
GABA: $y = 0.0423x + 0.0299$, $R^2 = 0.9967$

*Key: x – Concentration of amino acids (*μM*); y – Normalized area of the peak*

The concentrations of the amino acids were presented as means ± SEM. The extracellular amino acid levels in the dialysates were expressed in μM $15min^{-1}$. It was hypothesized that The concentrations of each amino acid were different among groups and there were differences at Day 2, 4, 7 and 14 compared with Day 1 after stroke in each group. The hypotheses were tested with repeated two-way ANOVA followed by Bonferroni post-hoc test.

3.5.2 Post-mortem analysis

The percentage of brain weight was calculated by Equation 1.

$$\%\,\text{Brain Weight} = \frac{Wt_{brain}}{Wt_{body}} \times 100\% \qquad \text{Equation 1}$$

Keys: Wt_{brain} – Weight of Brain
Wt_{body} – Body weight before sacrifice

Edema index, infarct index and actual infarct volume were calculated for each groups and were presented as means ± SEM. Edema index was obtained by Equation 2.

$$\text{Edema Index} = \frac{Vi}{Vc} \qquad \text{Equation 2}$$

Keys: V_i – Volume of hemisphere of ipsilateral side to MCAo
V_c – Volume of hemisphere of contralateral side to MCAo

Infarct index was calculated by the ratio of the infarct volume to the whole volume of cerebrum (Takamatsu et al 2002). Brain damage might be overestimated if there was edema after stroke. Therefore, if edema index was equal to or greater than 1 (cerebral edema), the brain damage was corrected with the edema index value by Equation 3.

$$\text{Brain damage} = \frac{\text{Infarct index}}{\text{Edema index}} \qquad \text{Equation 3}$$

The damage might be underestimated if there was atrophy. If edema index was smaller than 1 (cerebral atrophy), the brain damage was calculated with Equation 4 (Takamatsu et al 2002).

$$\text{Brain damage} = \frac{V_c - V_i + V_{in}}{V_c + V_i} \times 100 \qquad \text{Equation 4}$$

Keys: V_i – Volume of hemisphere of ipsilateral side to MCAo
V_c – Volume of hemisphere of contralateral side to MCAo
V_{in} – Infarction volume

Muscle weight was expressed by the ratio of wet weight of tibialis anterior (both affected and unaffected sides; with tendon) to the body weight before sacrifice.

Brain weight, edema index, infarct index and brain damage were presented as means ± SEM for each group. It was hypothesized that there were differences between groups in these parameters. Statistical differences between groups were determined by one-way ANOVA followed by Bonferroni post-hoc analysis. The hypotheses were that muscle weight of left tibialis anterior was greater in NMES group compared with other groups (differences between groups) and the muscle weight on the left side was higher than that on the right side. Muscle weight was tested with two-way ANOVA followed by Bonferroni post-hoc analysis.

CHAPTER 4 RESULTS

4.1 Pilot study

25 young male Sprague-Dawley rats (3 months) were used in the pilot study. It was found that rats with 40minutes and 60minutes showed mild neurological deficits from the postural tests (usually scored 1 in Longa's scale) and recovered (scored 0) within a few days. The TTC brain slices showed no infarction volume after 2 weeks. 40 minutes (n=9), 60 minutes (n=7), 75 minutes (n = 4), 90 minutes (n=2) and 120 minutes (n=3). 75min-Occlusion produced an observable infarction whereas 90-min and 120-min occlusion produced the most severe stroke. All rats died after 90min or 120min-occlusion within two weeks. Therefore, 75min-occlusion was determined as a suitable duration to produce considerable infarction with survival time over two weeks. This protocol was used in the whole study.

4.2 Study I: Evaluations of the effects of interventions based on assessment scores and stained brain slices

151 young male Sprague-Dawley rats (3 months) were used in this experiment. Ninty-six rats were excluded in this study, in which stroke was failed to be induced in thirty-eight rats (scored 0 in Longa's scale after recovering from anesthesia) and fifty-eight rats died of severe stroke or subarachonoid hemorrhage after middle cerebral artery occlusion. Fifty-five rats were randomly assigned into 4 groups as shown below:

Group	n
Sham-operated group (Sham group)	6
Control group (Control group)	17
Exercise group (EX group)	17
Electrical stimulation group (NMES group)	15

4.2.1 Body weight

Data obtained on Day 1, 2, 4, 7, 14 were taken for statistical analysis. As shown in Figure 4.1, mean change in body weight of Sham group generally increased throughout the two weeks and the increase reached a significant level at the end of the two weeks (p-value < 0.05). The body weight of Control, EX group and NMES group decreased rapidly in the first week. The loss in weight in Control group was significant in the first 2 days and then returned to original weight (weight on Day 0) at the end of the first week. Significant gain in weight was observed on Day 14. Rats in EX group kept losing weight throughout the 2 weeks and the weight returned to approximately the level before MCAo on Day 14. There was no significant loss or gain in weight in NMES group. The mean-change of body weight of NMES group increased a slight faster than that of EX group.

Sham group kept significantly higher mean change in body weight compared with the other three groups at most time points during the 1st week after stroke. There was no significant difference between the three groups (Control, EX and NMES groups) according to the post-hoc analysis at all the time points.

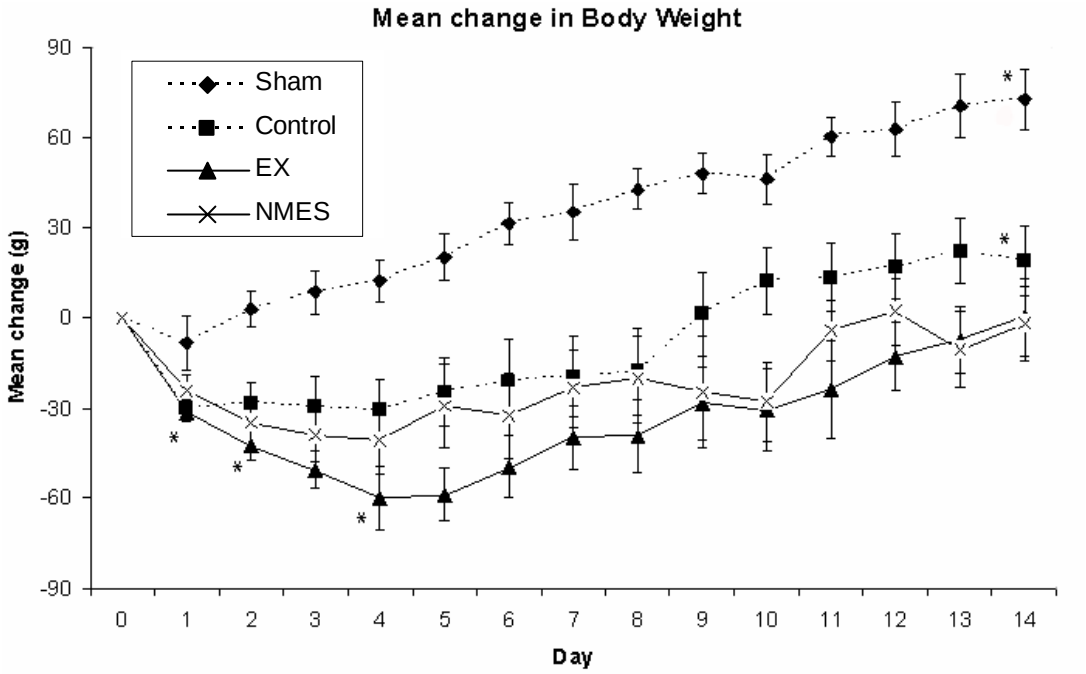


Figure 4.1 Mean-changes in body weight (grams ± SEM) before (Day 0) and after MCAo (Day 1-14) or sham operation. The day before MCAo is denoted as 'Day 0'. * $p<0.05$ compared with the weight on Day 0.

4.2.2 Neurological deficits scores from postural reflex test

Rats in Sham group scored 0 throughout two weeks. During the first week after MCAo, Control, EX and NMES groups exhibited similar level of neurological deficits, as shown in Table 4.1 and Figure 4.2. They decreased gradually during the two-week training, which indicated the recovery from neurological deficits. Control and EX groups improved in the neurological scores significantly after the first week and improvement continued throughout the second week, compared with the scores on Day 2 after MCAo (p-values<0.05 in post-hoc analysis). Control group improved in the greatest extent with a rapid pace in neurological deficits. NMES group showed no significant improvement throughout the two weeks. Starting from Day 8, the scores in control group kept at lower level than the two treatment groups.

However, no significant difference was found between groups throughout the two weeks.

Table 4.1 Neurological scores of all groups on Day 1, 2, 7 and 14. They were expressed as means (range).

Day after MCAo	Group			
	Sham	Control	Exercise	Electrical stimulation
1	0.000 (0-0)	1.135(1-2)	1.118 (1-2)	1.214 (1-2)
2	0.000 (0-0)	1.300(0-2)	1.353 (1-2)	1.333 (1-3)
7	0.000 (0-0)	1.071(0-3)	1.000 (1-1)	1.182 (1-2)
14	0.000 (0-0)	0.611(0-2)	0.800 (0-1)	1.000 (1-1)

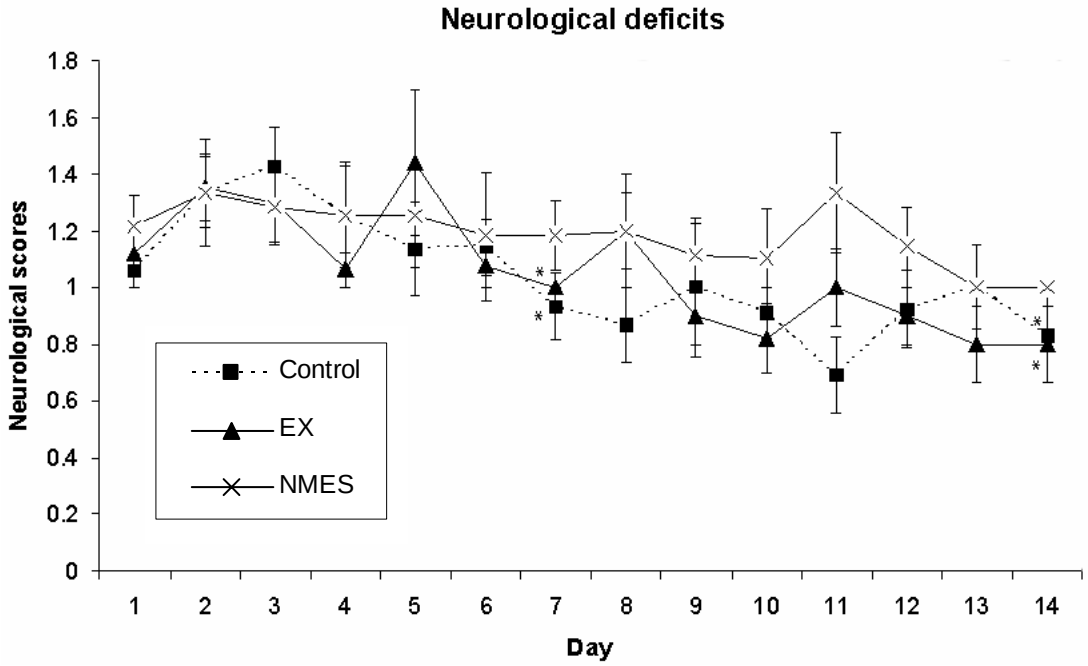


Figure 4.2 The changes in neurological scores (Longa's scale) of Control, EX and NMES groups over the 2 weeks (Sham group scored 0 throughout the 2 weeks and it is not shown in the above graph). Data are presented as means ± SEM. * $p<0.05$ compared with the scores on Day 1.

4.2.3 Limb placing tests

Limb placing scores of both affected and unaffected sides in Sham group were 16, which indicated that no deficit caused by stroke was present in this group. During

the first week, NMES group showed a significant increase in the scores (p-value = 0.45 on Day 7 compared with Day 1). However, on Day 14, the scores dropped to a lower level, which had no significant difference with the score on Day 1. Improvement of the scores in EX group was started from Day7 onwards whereas in Control group, the score was significantly improved on Day 14 (p-value = 0.006). ANOVA showed that significant difference between groups was only found on Day 14. On day 14, NMES group had a significantly lower scores than Control and EX groups (p-value = 0.006 and 0.017 respectively). Being consistent with the results in neurological deficit scores in Section 4.2, NMES group recovered slower than the other two groups. Regarding the unaffected side, the scores kept steadily around 14 to 16 over the 2 weeks in the three groups as shown in Figure 4.3b. No significant difference was found between or within groups.

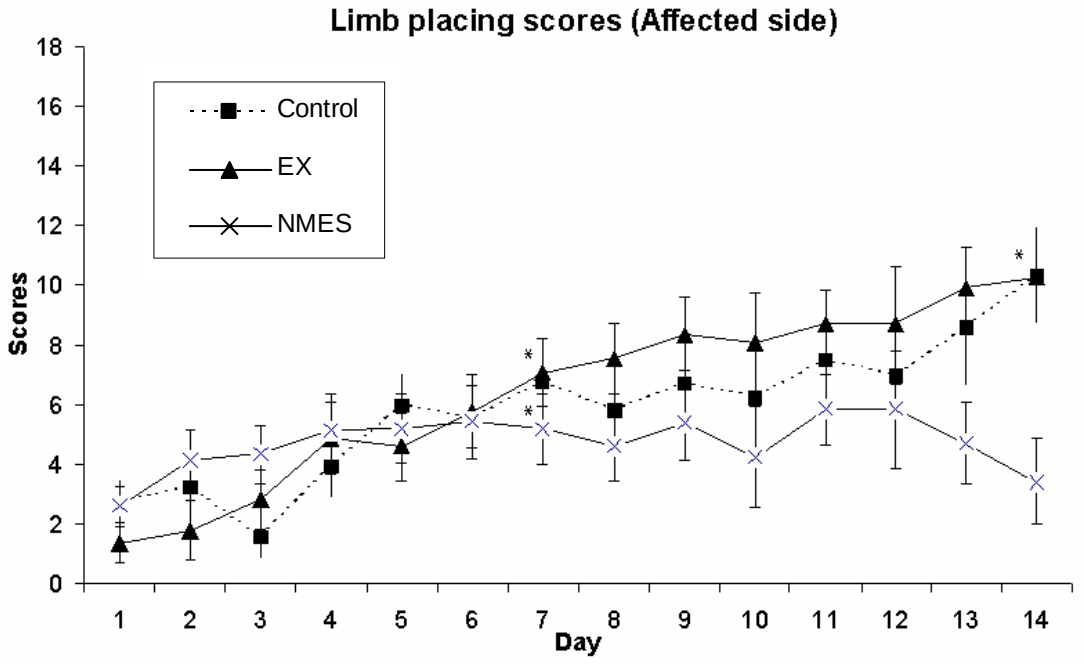


Figure 4.3 (a) The changes in limb placing scores (maximum 16) of Control, EX and NMES groups throughout the 2 weeks on the affected side. Data are presented as means ± SEM. * $p<0.05$ compared with the scores on Day 1.

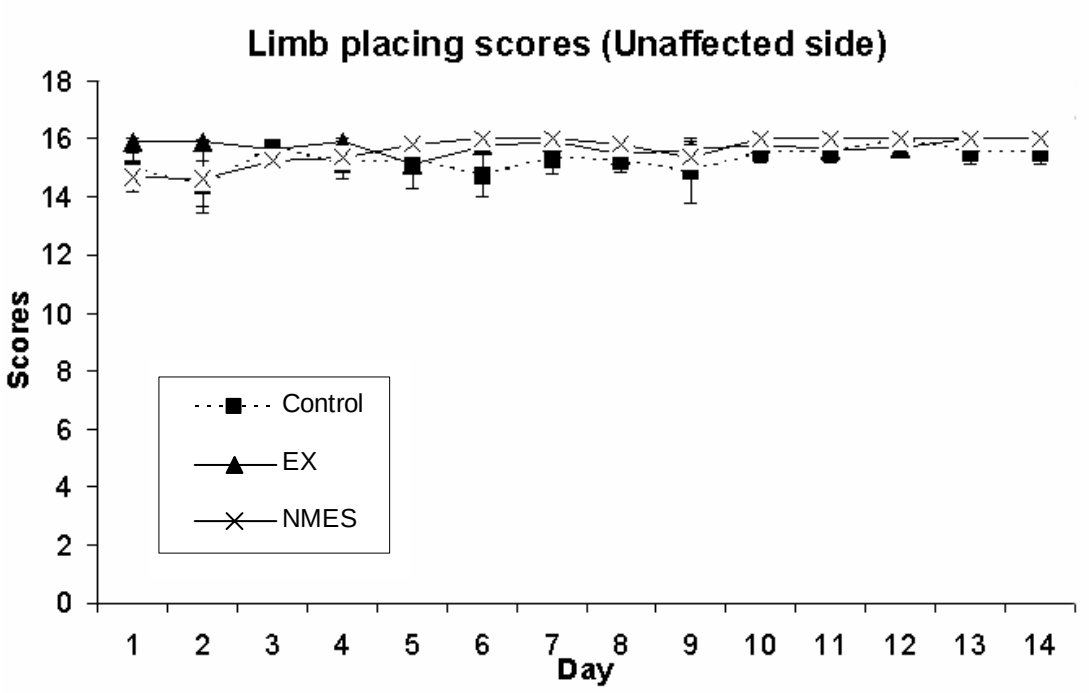


Figure 4.3 (b) The changes in limb placing scores (maximum 16) of Control, EX and NMES groups throughout the 2 weeks on the unaffected side. Data are presented as means ± SEM.

4.2.4 Percentage of brain weight

According to Figure 4.4, the percentage of brain weight was smallest in Sham group, followed by the Control group. Among the two treatment groups (NMES and EX groups), the percentage of brain weight was larger than the NMES group with no significant difference. The differences between groups did not reach significant level (p-value = 0.063).

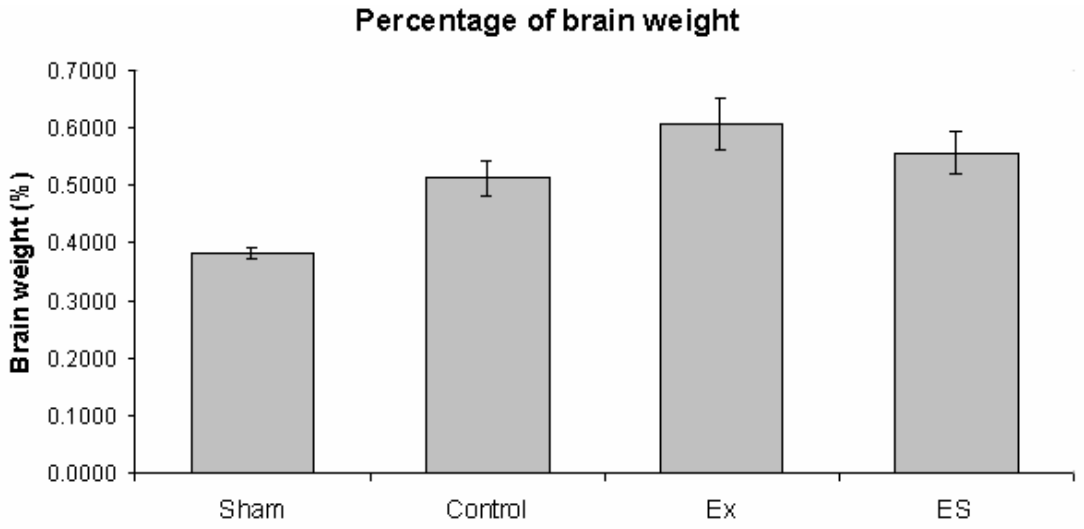


Figure 4.4 The percentage of brain weight of Sham, Control, EX and NMES groups. Data are presented as means ± SEM.

4.2.5 Muscle weight

The percentage of muscle weights of tibialis anterior on both affected and unaffected side were a bit larger in NMES group, compared with the other three groups. Moreover, the difference in weight between the affected and unaffected side was greater in NMES group, in which the tiabialis anterior on the affect side was heavier than the unaffected side. On the affected side, the muscle weight of NMES group was significantly greater than the Control and Sham groups (p-value = 0.049 and 0.017 respectively). On the unaffected side, the muscle weights had no significant difference between groups. In the other three groups, the weights of muscles on the affected and unaffected sides were similar with no significant difference (p-value = 0.483).

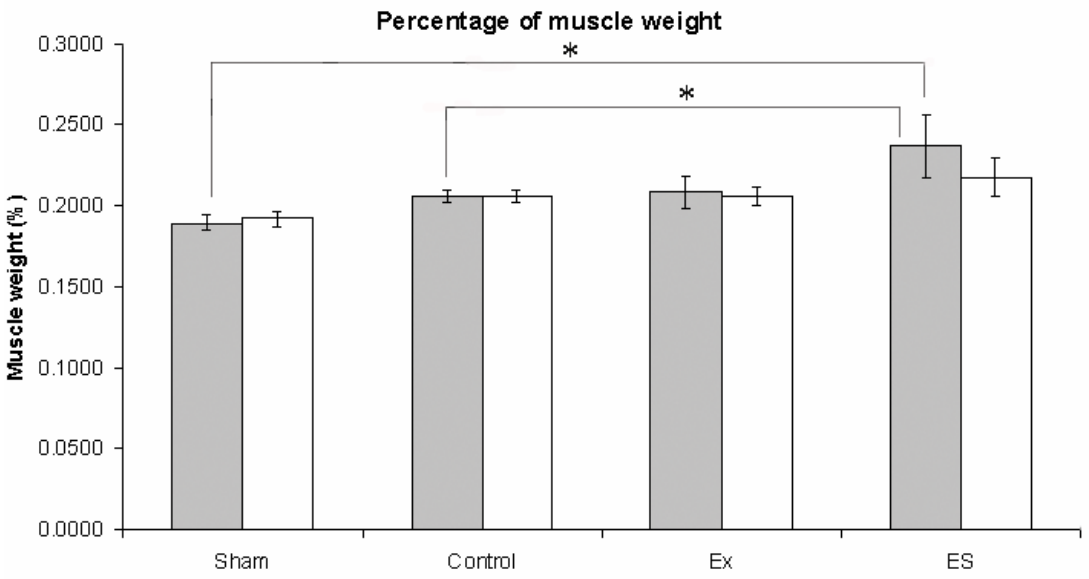


Figure 4.5 The muscle weight of tibialis anterior on the affected (shaded bars) and unaffected sides (white bars). Data are presented as means ± SEM. * $p<0.05$ compared between NMES, Control and Sham groups

4.2.6 Actual infarction volume, infarct index and brain damage

The regions and extents of infarction of the four groups were illustrated in the TTC-stained brain slices as shown in Figure 4.6. The lighter area (white in colour) indicated the ischemic area (infarction) while the darker areas (red in colour) were the healthy brain tissue. No infarction was induced in Sham group (actual infarct volume = $0mm^3$). Some brain slices had missing part in the infarct area, which was due to necrotic tissue developed throughout the two weeks after stroke. According to Table 4.2, the actual infarct volume of the NMES group was the smallest whereas the EX group was a bit larger than the Control group. However, after normalization with the body weight before sacrifice, Control group had a greater than the EX group and the normalized infarct index of NMES group was still the smallest (Figure 4.7). Both the actual infarct volume and normalized infarct index did not showed significant differences between groups (p-value = 0.320, 0.287 respectively).

Brain damage was calculated by eliminating the edema factor, which could affects the measurement of brain volume considerably. Control group had larger brain damage compared with Sham, NMES and EX groups. Significant difference was found between groups (p-value=0.047), but post-hoc analysis showed no significant group differences. (p-values = 0.16 (Control - Sham), 0.22 (Control - NMES), 1.00 (Control - EX)).

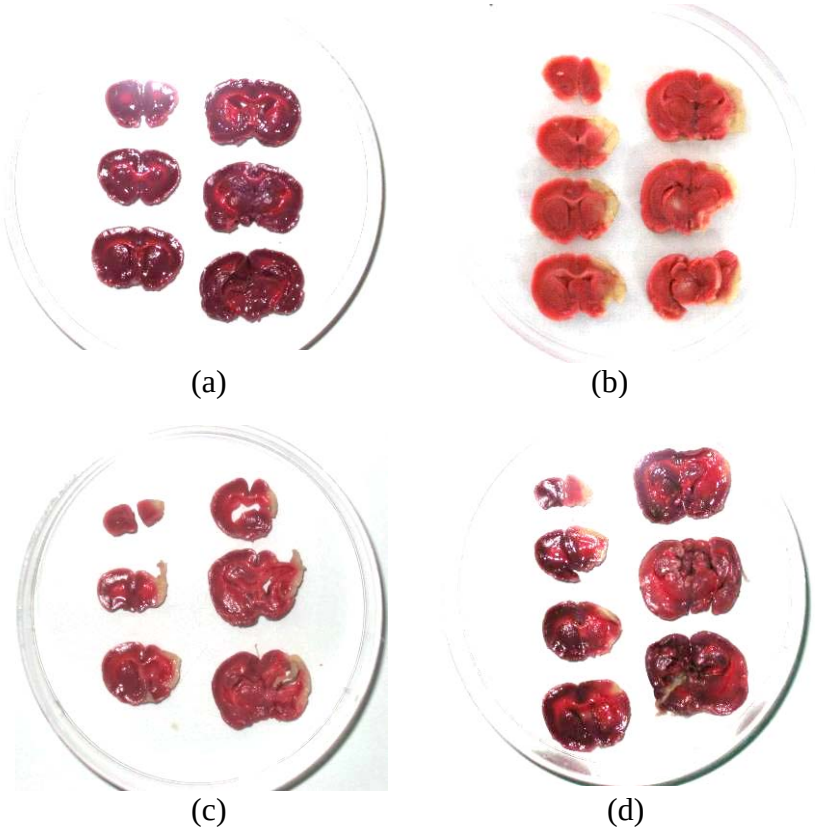


Figure 4.6 The TTC-stained brain slices (the first six slices from cerebrum) showing the infarction region and extent of different groups: (a) Sham-operated; (b) Control; (c) Exercise; (d) Electrical stimulation

Table 4.2 Actual infarct volume, normalized Infarct index and brain damage of the Sham, Control, EX and NMES groups. Data are presented as means ± SEM.

Group	Actual Infarct volume (mm^3)	Infarct index	Brain damage
Sham	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Control	78.929 ± 15.960	0.065 ± 0.013	11.498 ± 1.675
Exercise	81.851 ± 25.880	0.062 ± 0.019	8.836 ± 1.728
NMES	37.003 ± 13.254	0.028 ± 0.010	5.107 ± 1.932

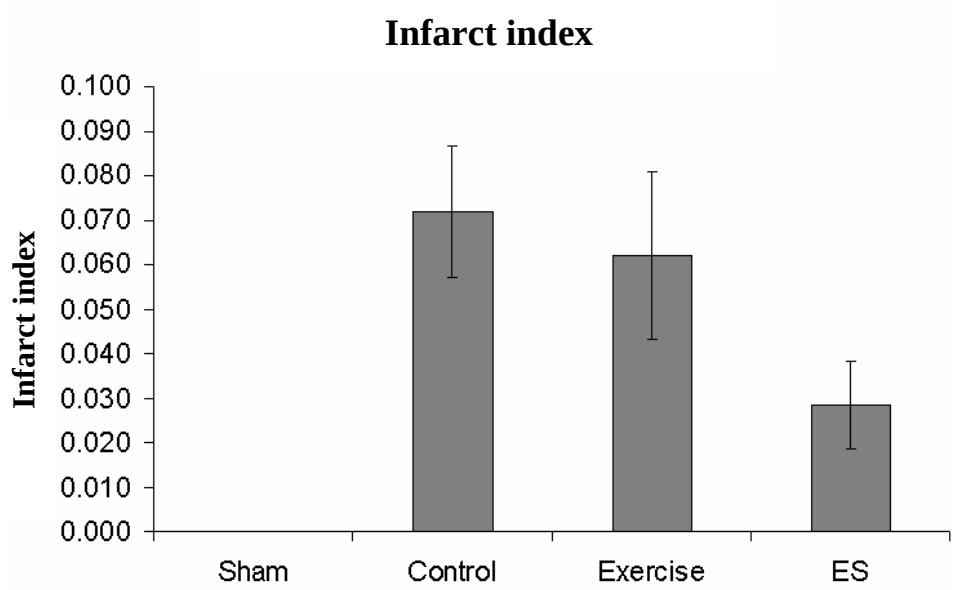


Figure 4.7 Infarct indexes of Sham (no infarction), Control, EX and NMES groups. Data are presented as means ± SEM.

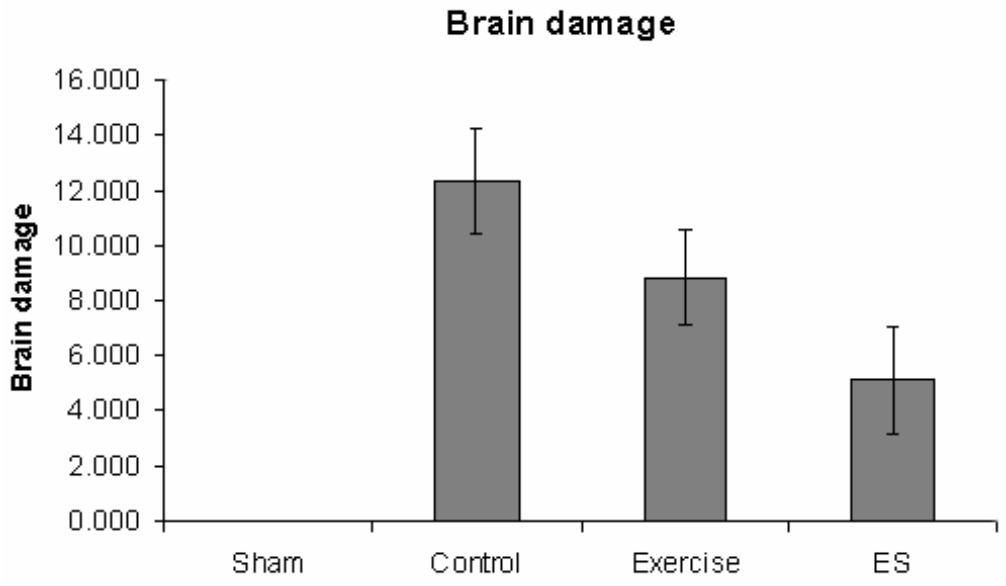


Figure 4.8 Brain damages (corrected with edema index) of Sham (no infarction), Control, EX and NMES groups. Data are presented as means ± SEM.

4.2.7 Edema index

Edema index refers to the ratio of ipsilesional hemisphere volume to contralesional hemisphere volume. Therefore, edema index greater than 1 indicates edema in the ipsilesional hemisphere, while if it is smaller than 1, atrophy should be observed in the lesioned hemisphere. There was no atrophy or edema occurred in Sham group (edema index = 1). The atrophy in Control group was the most severe among the four groups, followed by the EX group. Lesioned hemisphere in NMES group were also atrophied, but with a lesser extent compared with Control and EX groups (Figure 4.9). However, the differences between groups did not reach a significant level (p-value = 0.181).

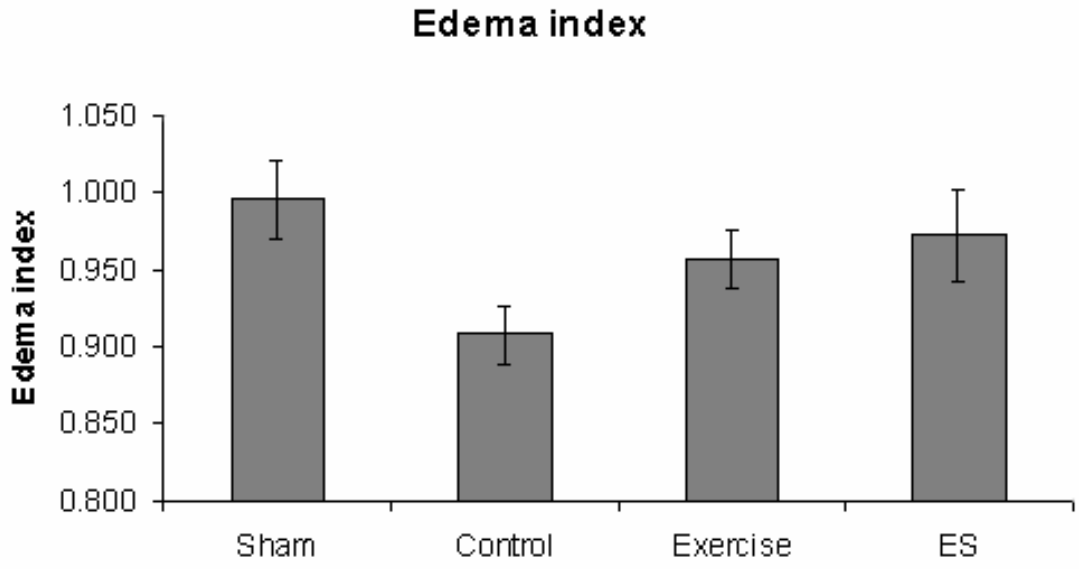


Figure 4.9 Edema indexes of Sham, Control, EX and NMES groups. Data are presented as means ± SEM.

4.3 Study II: Microdialysis study

48 young male Sprague-Dawley rats (3 months) were used. Only 19 rats were included in the study based on the postural tests after recovering from anesthesia. Others were either dead or failed to induce stroke after MCAo. The 19 rats were

randomly assigned into three groups - Control (n=6), EX group (n=8) and NMES group (n=5).

4.3.1 Concentration of aspartate

Aspartate concentrations in EX and Control groups exhibited similar trend and levels. The levels fluctuated throughout the two weeks. In NMES group, the aspartate level reached the peak on Day 2, but with no significant difference with the other days. There were no significant differences between groups or between days in all groups at all time points.

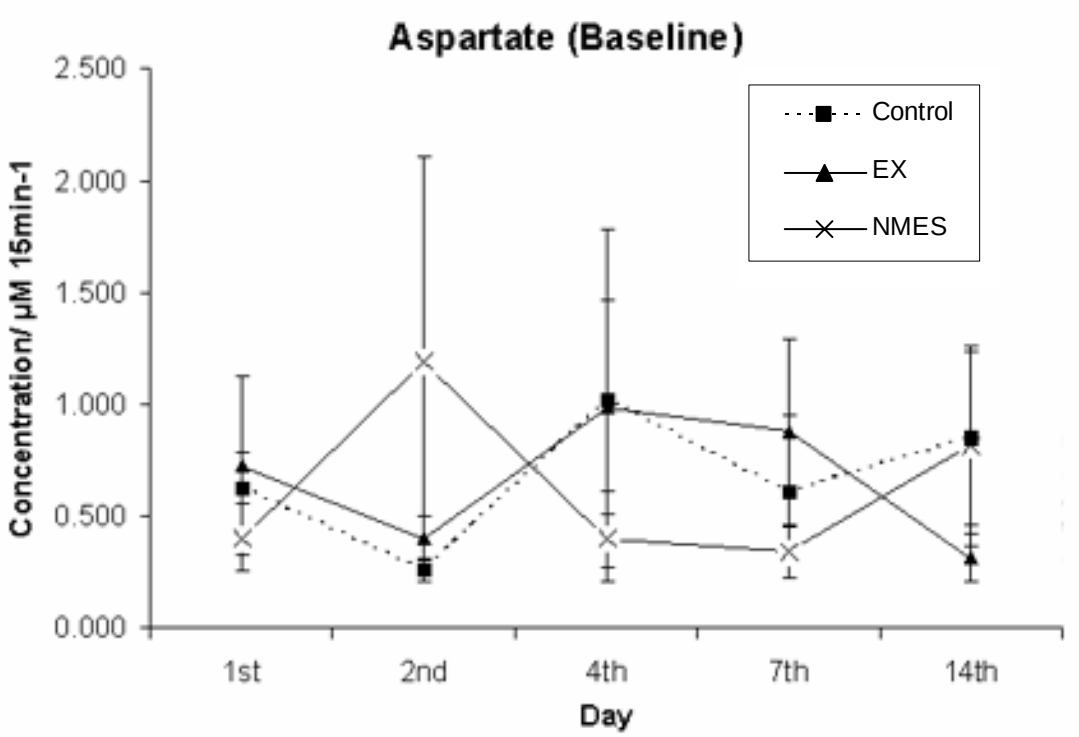


Figure 4.10 Basal concentrations of aspartate of NMES group, Control group and EX group over the 14 days. The concentration is expressed in µM $15min^{-1}$ ± SEM.

Figure 4.11 showed the changing level of hippocampal aspartate of NMES, EX and Control on Day 4. The concentration at time point 'Baseline' represented the

average concentration of samples taken during the hour before intervention, while at 'Intervention', it referred to the average concentration of samples taken during the 30-min treadmill exercise or neuromuscular electrical stimulation. Concentration at '1hr-Post-ex' was the average concentration during the first hour after intervention whereas '2hr-Post-ex' was the average during the second hour. Rats in Control group remained inactive on the non-running treadmill at all time during the sample collection. Aspartate concentration in Control and NMES groups kept at relatively low level at all time points. EX group increased during intervention and then returned to baseline level at 1-hr- and 2-hr-Post ex. However, there was no significant difference between groups or between time points within group.

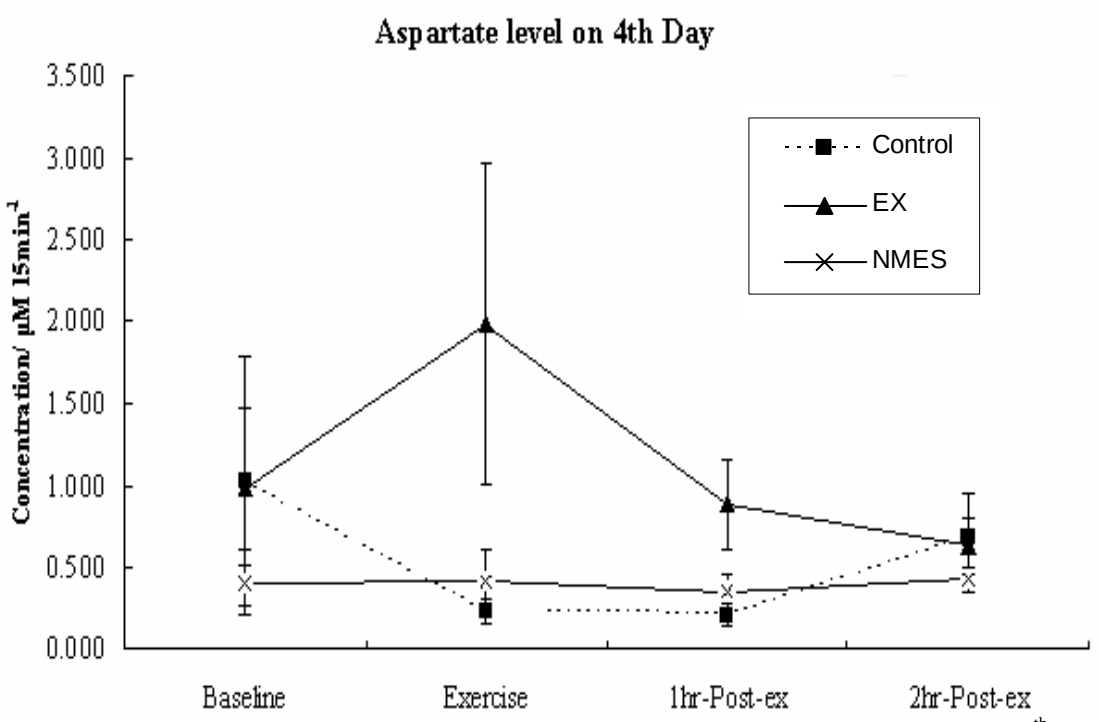


Figure 4.11 The change of aspartate level of NMES group, Control group and EX group on the 4th Day after MCAo. The concentration is expressed in µM $15min^{-1}$ ± SEM.

4.3.2 Concentration of glutamate

The concentrations of glutamate in NMES and EX groups increased to the highest levels at the 4th and 7th day respectively, and then dropped rapidly after reaching the peaks (Figure 4.12). Glutamate level in Control group was decreased from the 1st day after inducing stroke to the 14th day (1.56 to 0.71 μM $15min^{-1}$). There was no significant difference between days in each group at all time points and the glutamate concentration between groups did not reach significant level at all days.

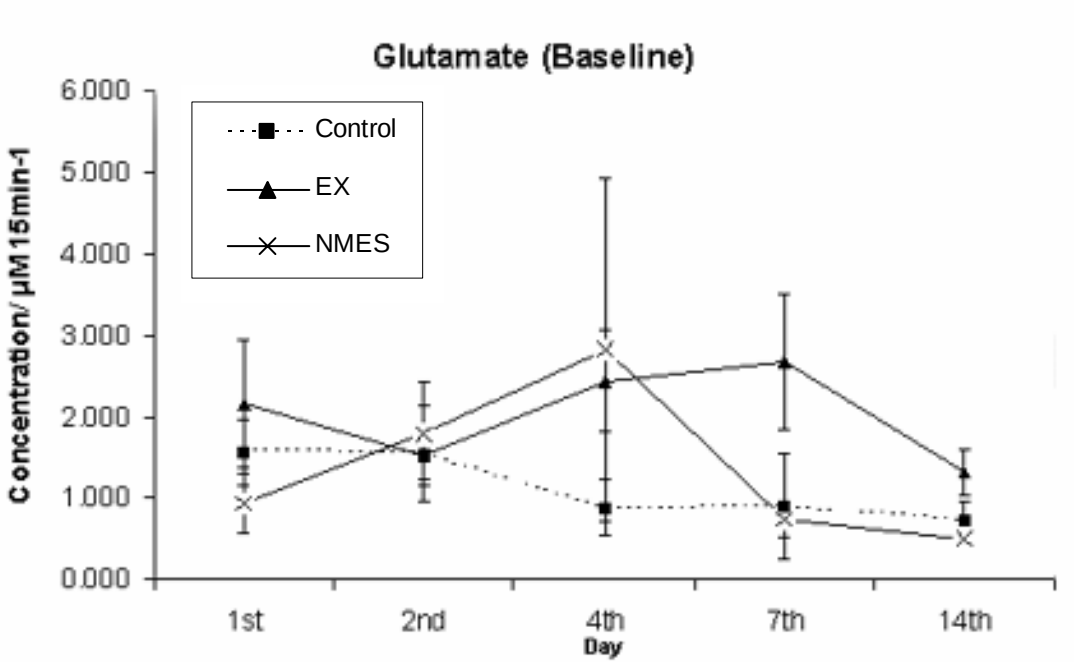


Figure 4.12 Basal concentrations of glutamate of NMES group, Control group and EX group over the 14 days. The concentration is expressed in μM $15min^{-1}$ ± SEM.

Hippocampal glutamate kept at a steady and low level at all times in Control group. In NMES group, the intervention seemed to have no effect on the glutamate release. Significant increase in glutamate (compared with baseline) was observed in EX group during intervention (p-value =0.35).

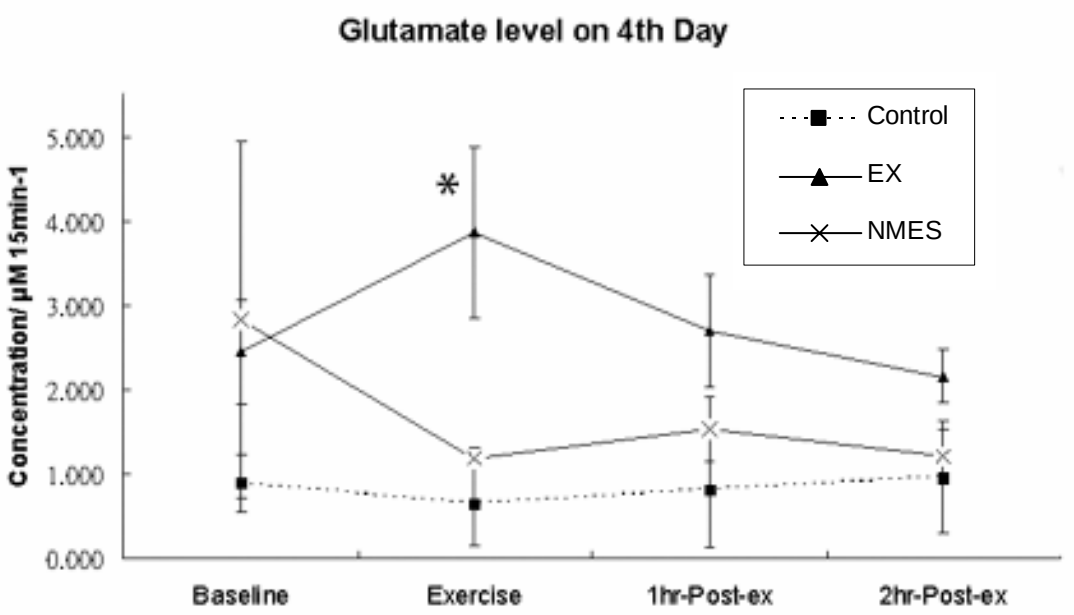


Figure 4.13 The change of glutamate level of NMES group, Control group and EX group on the 4th Day after MCAo. The concentration is expressed in µM $15min^{-1}$ ± SEM. * $p<0.05$ compared between a particular time point with baseline level in a particular group

4.3.3 Concentration of taurine

The basal concentration of taurine of rats in Control group dropped after reaching a peak at the 4th day, whereas the concentrations in EX and NMES groups showed an increasing trend over time after the 4th day (Figure 4.14). The level of taurine in EX group kept at a low and steady level (11.67 to 13.33 µM $15min^{-1}$), compared with NMES (11.27 to 17.19 µM$15min^{-1}$) and Control groups (18.932 to 22.012 µM$15min^{-1}$), throughout the intervention period (2 weeks). However, taurine level showed no significant difference over the two weeks in all groups. On Day 4, ANOVA showed significant group differences (p-value = 0.003), in which taurine concentration in Control group was significantly higher than that in EX and NMES groups (p-values = 0.002 and 0.012 respectively) according to the post-hoc test.

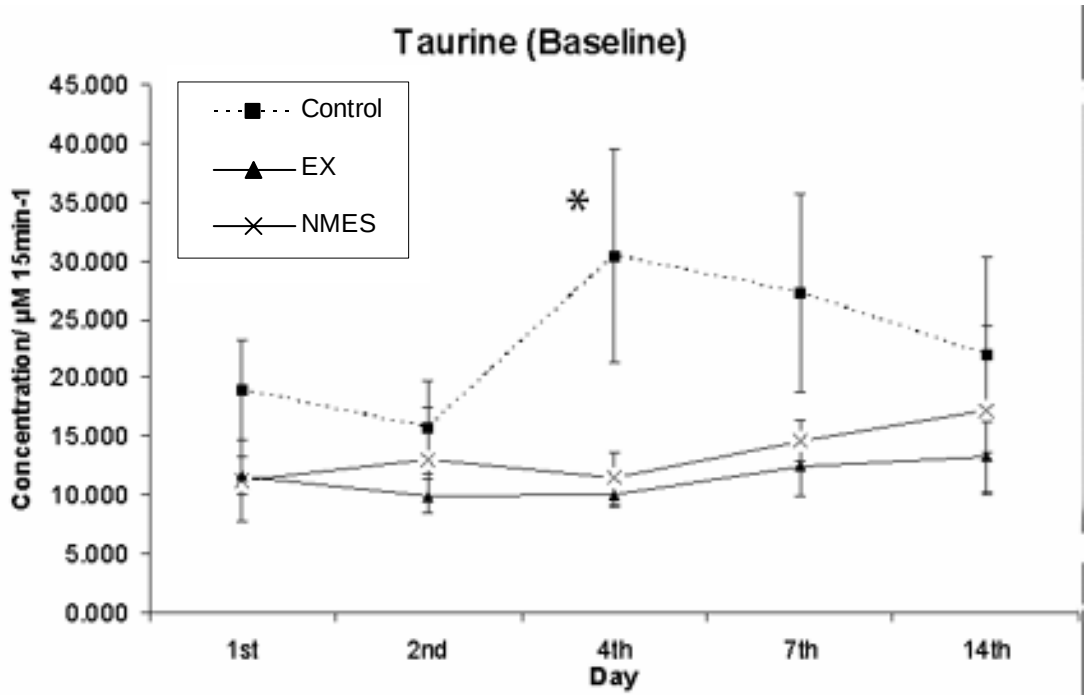


Figure 4.14 Basal concentrations of taurine of NMES group, Control group and EX group over the 14 days. The concentration is expressed in µM $15min^{-1}$ ± SEM. * $p<0.05$ compared between NMES, Control and EX groups

Figure 4.15 showed the changing level of hippocampal taurine of NMES, EX and Control on Day 4. The level of taurine decreased significantly after intervention in NMES group (p-values = 0.024 (1hr-Post-ex) and 0.016 (2hr-Post-ex), relative to Baseline). There was an increase in taurine level during intervention in EX group, but with no significant difference compared with the baseline (p-value = 0.315). The taurine level of Control group suddenly dropped with a large extent (38.50 to 25.01 µM $15min^{-1}$) during the intervention time slot, in which the rats were remained in the cage without any interventions. Significantly higher taurine levels were found in Control group, comparing with NMES and EX groups, at baseline and the second post-ex time pointes with p-values = 0.003 (Baseline) and 0.031 (second post-ex).

Post hoc analysis showed significant differences between Control and both groups at baseline and the second post-ex time point.

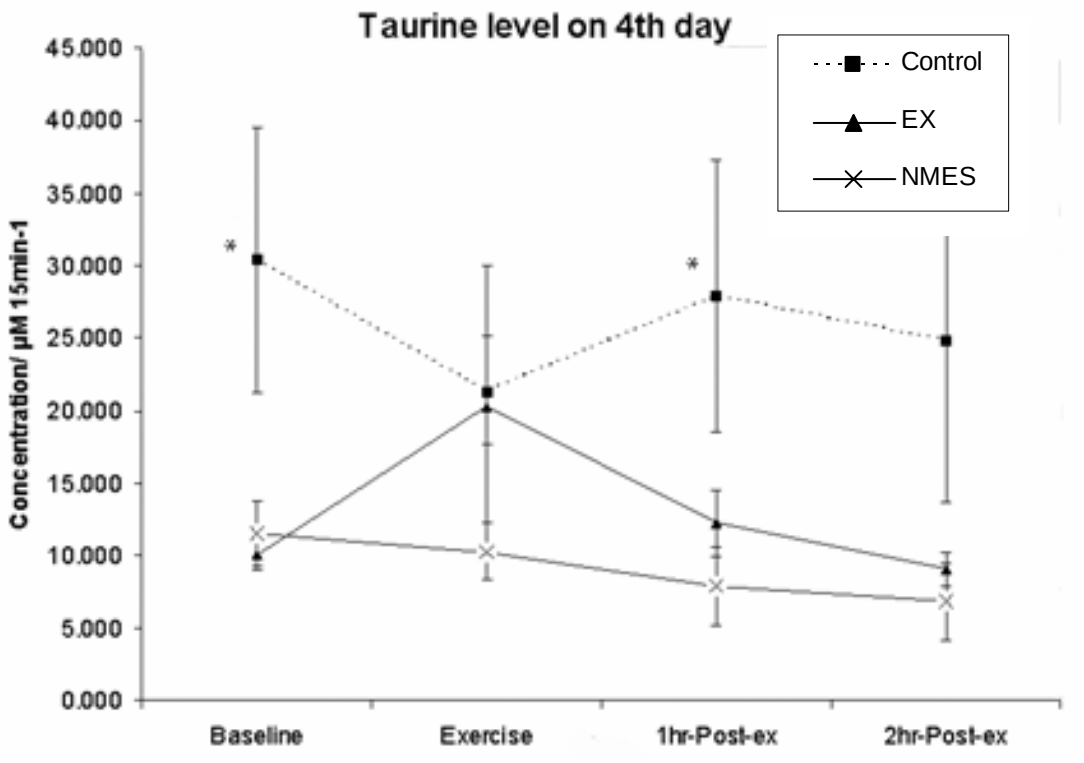


Figure 4.15 The change of taurine level of NMES group, Control group and EX group on the 4th Day after MCAo. The concentration is expressed in μM $15min^{-1}$ ± SEM. * $p<0.05$ compared between NMES, Control and Sham groups

4.3.4 Concentration of GABA

In Control group, GABA concentration in hippocampus increased gradually and reached the peak at the 7th day and then dropped on the 14th day (Figure 4.16). Although the GABA levels in EX group kept higher than that in NMES group, no significant difference was found among the three groups. The levels in both EX and NMES groups reached the peak on the 4th day. No significant difference between days in each group at all time points

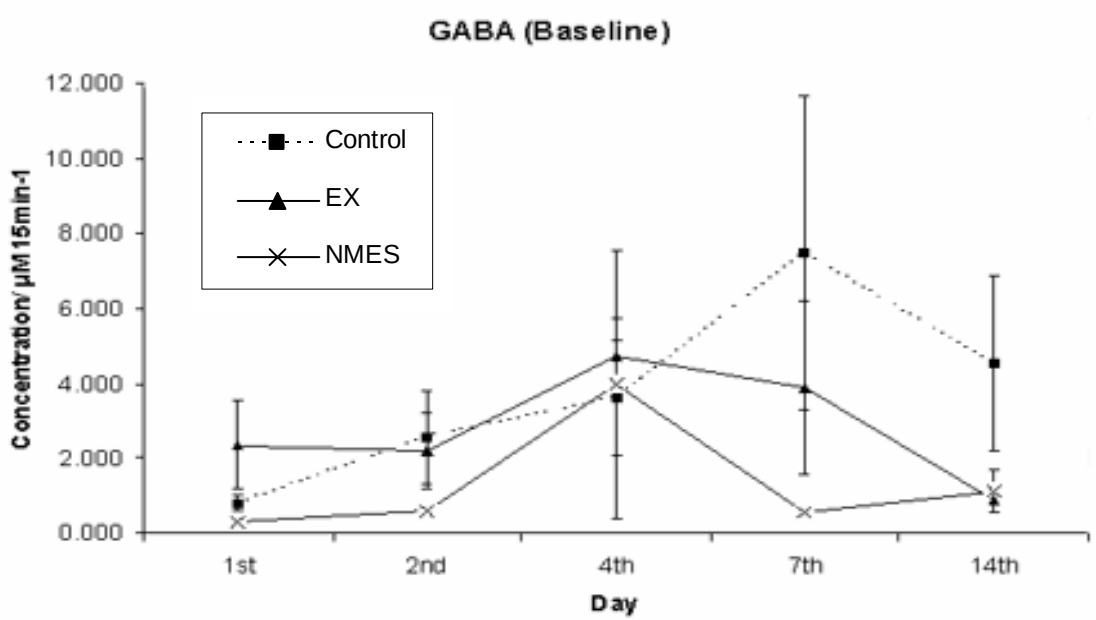


Figure 4.16 Basal concentrations of GABA of NMES group, Control group and EX group over the 14 days. The concentration is expressed in µM $15min^{-1}$ ± SEM.

Hippocampal GABA in Control group exhibited a steady trend and kept at low levels at all time points. The level in EX group decreased with time whereas in NMES group, the concentration of GABA increased one hour after intervention. There was no significant difference between groups or between time points within group.

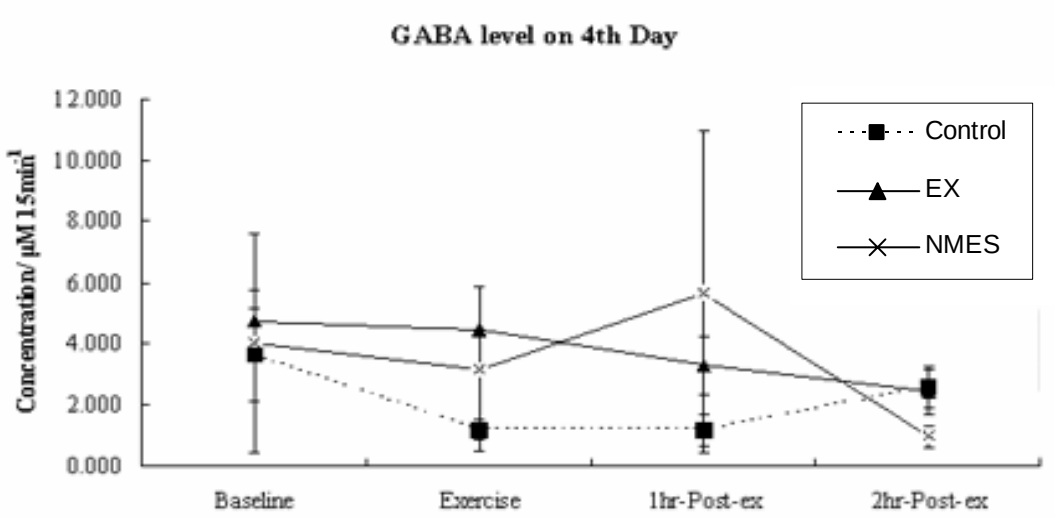


Figure 4.17 The change of GABA level of NMES group, Control group and EX group on the 4th Day after MCAo. The concentration is expressed in µM $15min^{-1}$ ± SEM.

CHAPTER 5 DISCUSSION

This study demonstrated the effects of treadmill exercise and neuromuscular electrical stimulation on adult rats following focal cerebral ischemia in neurological, neurochemical and physiological aspects. The first part of this chapter was focused on some important issues regarding the methodology. Then, the physiological changes induced by monofilament middle cerebral artery occlusion followed by reperfusion were discussed. In addition, the effects of treadmill exercise and neuromuscular electrical stimulation on stroke recovery were examined based on the results presented in Chapter 4. It was followed by the neurochemical changes caused by cerebral ischemia and interventions. Efficacies of these two interventions were compared in the physiological aspect. Finally, findings about critical time for intervention after stroke and possible mechanisms of recovery were discussed.

5.1 Methodological aspect

5.1.1 Intraluminal suture techniques

An important inclusion criterion of rats in this study was that they had to score at least 1 in postural reflex test at the first two days after stroke. Approximately one-fourth of the rats failed to meet this criterion and they were regarded as failure case and were excluded from all analysis. Failure of inducing stroke by intraluminal suture method could be explained by the different diameters of sutures used as the intraluminal suture, different pattern of blood circulation in middle cerebral artery area by collateral arteries among individuals and time of occlusion. Nylon suture

with size 4-0 was used at the beginning of this study, as many researches in literatures did (Longa *et al* 1989; Koizumi *et al* 1996; Li *et al* 1999). However, it was found that the successful rate was very low. Induction of stroke became more successful when Nylon suture with larger diameter (3-0) was used. Kuge *et al* showed in their study that slight differences in diameter significantly affected lesion volume in stroke rat model using intraluminal suture technique (Kuge 1995). Although no further investigation on the anatomy of the relevant arterial system in this study, some researchers did demonstrated that these individual patterns considerably affected the lesion size (Rubino 1988). Time of occlusion affected the pattern of behavioral deficit and regions of infarction of the brain (Belayev 1996; Aronowski 1994). Several durations of occlusion were tried in the pilot study (see Sections 3.2 and 4.1 for details). Among 40-, 60-, 75-, 90- and 120-minutes occlusion, 75-minutes was found to be the most suitable one, in which considerable infarction was produced and consistent neurological deficits were observed throughout the two weeks. Stroke produced by 40- and 60-minute occlusion was very mild and stroke by 90- and 120-minute were too severe and mortality rate was high.

Nearly one-third of the rats were dead within 48 hours after middle cerebral artery occlusion. In most cases, hemorrhage was observed at the circle of Willis upon dissection and it was suspected that the subarachnoid hemorrhage resulted from excessive insertion pressure of the intraluminal filament (Garcia 1993).

5.1.2 In vivo sampling by microdialysis

Intracerebral microdialysis is a useful method for studying dynamic chemical changes in the extracellular fluid. However, microdialysis cannulae cause a certain degree of damage to brain tissue. After inserting the cannula, there is an initial period of disturbed tissue function involving increased glucose metabolism and decreased cerebral blood flow, but this situation returned to normal after 24 hours (Benveniste 1987). Astrocytic hypertrophy and axonal damage with anterograde and retrograde degeneration were observed and gliosis was formed by the third day of cannula implantation. The astrocytes were gradually replaced by connective tissue after two weeks (Benveniste and Diemer 1987; Shuaib *et al* 1990). Figure 5.6 showed the gliosis on the cortex of the ipsilesional hemisphere caused by the long-term implantation of cannula (more than 2 weeks after implantation). In our study, microdialysis sampling was commenced at least 3 days after implantation of cannula. Although this might minimize the influences due to the tissue changes, several factors affecting the long-term microdialysis must be considered. These factors included biochemical alterations due to histological changes, coating of the probe with inflammatory cells and connective tissue, changing diffusion characteristics of the probe and tissue, and the loss of integrity of the blood-brain-barrier (Khan and Shuaib 2001). Therefore, the levels of neurotransmitters measured in this study might also be affected by these changes. However, they are still important indexes for comparing the effects of different interventions on the ischemic damaged brain. Moreover, it was noted that neurological deficit or extent of ischemic damage were not affected by any procedures in this microdialysis study.

Thus, in vivo microdialysis sampling is an important and reliable tool for evaluating the neurochemical changes without disturbing the changes due to ischemic injury.

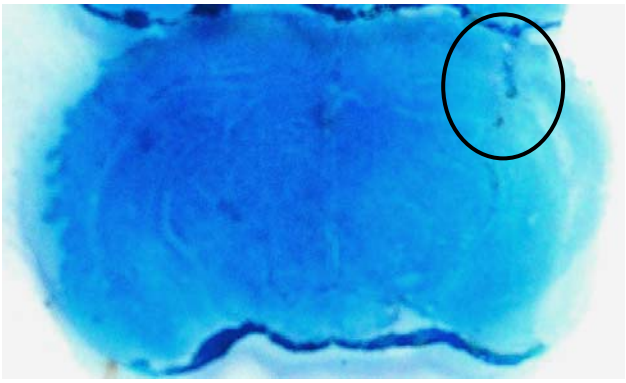


Figure 5.1 Location of the cannula on the brain. The brain slice was dyed with Evan's blue

5.1.3 Variability of infarction volume

Brain temperature affects the extent of ischemic brain damage. Hypothermia had a protective effect (Moyer *et al* 1992) and hyperthermia had an aggravating effect (Morikawa *et al* 1992). In our study, the body temperature of the rat was kept constant using a heating lamp during surgeries. Blood glucose level is another factor affecting the brain damage during cerebral ischemia. Hyperglycemia might increase infarction by exacerbating tissue acidosis whereas under hypoglycemia, there is insufficient energy substrate for the brain (Warner *et al* 1995). Another factor modulating the severity of ischemic brain injury is oxygen saturation. A low oxygen partial pressure speed up the occurrence of necrosis in penumbra area since oxygen supply would be inadequate to maintain cell viability under the condition of reduced cerebral blood flow (Watson *et al* 1997). Blood pressure could also affect the brain infarction. During focal cerebral ischemia, local cerebral blood flow is directly

dependent on perfusion pressure and arterial pressure. A decrease pressure could compromise the residual flow in penumbra area and contribute to the extensive infarction (de Lecinana *et al* 2001). In further study, it is recommended that these parameters should be closely monitored during surgery of MCAo.

5.1.4 Clinical relevance of used ischemia models

It is no doubt that no animal model can exactly mimic stroke in human. Some limitations must be considered such as different surgical techniques involved in occlusion of the artery, differences in the size and variability of the ischemic lesion at different laboratories. Moreover, these models mimic at best less than 25% of all strokes (Small and Buchan 2000). Important factors which contributed to the low resemblance of animal models to human stroke included ages and health status. Usually, young and healthy animals are used to carry out the experiments, while in reality, cerebrovascular diseases usually occurs in aged population. Additional diseases and risk factors, which were always observed in aged population, could not be ignored. Despite the limitations, animal study is still an important tool to study cerebral ischemia and mechanisms of stroke and motor recovery, to investigate the efficacy of treatment such as medication and physical exercise. In this study, timing of intervention, extent and location of ischemic brain damage, natural history of recovery, sex and age, and levels of activity were well-controlled to ensure the homogeneity of the population, and thus a reliable comparison (Dobkin 2004). Neurological deficits, intracerebral microdialysis and post-mortem analyses were used to determine the efficacy of treadmill exercise and neuromuscular electrical

stimulation in stroke rat. These measurements were difficult or impossible to carry out in human subjects. Results from microdialysis was highlighted in this study since the restoration of neurotransmission in spared tissue near or remote from an infarction has long been an interest regarding the motor recovery after stroke (Dobkin 2005).

5.2 Physiological and neurological changes due to cerebral ischemia

Body weight is an important measure to evaluate the general physiological condition and recovery of experimental rats after MCAo (Dittmar *et al* 2003). Its development was correlated to histopathologically observed ischemic brain damage (Palmer *et al* 2001). In our study, reduction of weight was only observed in groups with cerebral ischemia (Control, EX and ES groups) while Sham group continuously gained weight 2 days after surgery. It could be explained by previous study, which stated that the ligation of external carotid artery in intraluminal filament model of cerebral ischemia led to pain and impairment of mastication and swallowing in rats, thereby resulting in reduced food and water consumption (Dittmar *et al* 2003). Other factors contributing to the weight loss after stroke were anorexia and reduction of nutritional intake due to functional disability of upper arm (Choe *et al* 2004). Consequently, body weight kept losing in the first few days and it took a longer time for them to regain the baseline body weight.

Neurological deficits induced by focal cerebral ischemia are always characterized by sensorimotor dysfunction, these have been noted by previous investigators

(Bederson *et al* 1986; Markgraf *et al* 1992). Postural reflexes test and limb placing tests appeared to be particularly sensitive in detecting deficits after MCAo (Belayev *et al* 1996). In the present study, performance in postural reflexes test and limb placing tests was found to be improved significantly in Control group over the two weeks. Such improvement might be due to spontaneous recovery following ischemic injury of the brain, which was supported by previous studies (Yang *et al* 2003; Bland *et al* 2001; Belayev *et al* 1996). Spontaneous recovery might improve neurological performance by reducing infarction of the ischemic brain, increasing excitability in both lesioned and non-lesioned cortices, or increasing cell proliferation through neurogenesis induced by ischemic injury. In Yang *et al*'s study, the infarct volume was larger in the rats sacrificed 24 hours after middle cerebral artery occlusion than those sacrificed 2 weeks later without any intervention (Yang *et al* 2003). Human brain mapping studies revealed a greater than normal activity in ipsilateral and contralateral cortices in the early stage after stroke and a shift in brain activity toward more normal function in the later stage after stroke (Schaechter 2004). Although brain had long been regarded as a non-regenerating organ, accumulating evidences showed neurogenesis occurred in several regions of the brain in the first few weeks after ischemic injury, which suggested a large capacity for self-repair in the injured brain (Parent *et al* 2002). However, it was still controversial that the functional outcome was correlated with neurogenesis induced by cerebral ischemia.

Brain weight of Control group was higher than Sham group, but without significant difference. This increase in brain weight might represent increase in blood vessels

(angiogenesis), glia, neuron soma size, dendritic elements and synapses (neurogenesis) (Kolb and Whishaw 1998). Angiogenesis, the new formation of blood vessels by sprouting from preexisting vessels, can occur after focal brain ischemia. It has been shown that active increased microvessel density developed in the penumbral area n both patients with cerebral stroke and in experimental stroke in rats (Krupinski *et al* 1994, Wei *et al* 2001). Neurogenesis was observed in several regions of the brain following cerebral ischemia (Felling and Levison 2003; Kee *et al* 2001; Jiang *et al* 2001). Takasawa *et al* reported that this kind of cell proliferation reached the peak at 4 days in the ipsilateral subgranular zone of the brain after ischemic insults and there was eightfold increase in cell proliferation at 7 days compared with sham-operated controls. Neurogenesis was not only observed in ipsilateral hemisphere, contralateral subgranular zone also exhibited a fourfold increase in cell proliferation compared with sham controls and reached the peak at 7days after ischemia (Takasawa *et al* 2002). Apart from subgranular zone, focal cerebral ischemia stimulated neurogenesis in subventricular zone (Jin *et al* 2004), which peaked at 7 days and persisted at least to 14 days after insults (Takasawa *et al* 2002). Persistent neurogenesis was also observed in hippocampus (Kee *et al* 2001), cortex and striatum (Jiang *et al* 2001) in adult rats after transient middle cerebral artery occlusion. Ischemia-induced neurogenesis in these regions is a potential source of new neurons after injury. The cells proliferated during ischemia could survive over considerable periods and differentiate into mature cells (Felling and Levison 2003). Previous study showed that the numbers of astrocytes in brain regions near the ischemic injury was markedly increased and chains of neuroblasts

extended to the injured striatum from the subventricular zone after focal ischemia (Parent *et al* 2002). These newly generated nerve cells induced by cerebral ischemia may contribute to the increase in brain weight. However, the functional significance of these cells was still unknown.

The brains in Control group were atrophied whereas no edema or atrophy occurred in Sham group. Moreover, infarction and necrotic tissues were observed 2 weeks after ischemia in Control group. These parameters verified the ischemic damage caused by the occlusion of middle cerebral artery by monofilament suture. Brain atrophy in rats 14 days after focal cerebral ischemia was reported by Takamatsu *et al*, as shown in Figure 5.1. The edema formation impairs microcirculation and thus brain function. This could be supported by the findings regarding the performance of the neurological scores, in which significant improvement was started on Day 7 after ischemic insults. An irreversible cell death was observed after focal cerebral ischemia. This is followed by the digestion of necrotic cells in the injured area and subsequent substitution of these cells with fibroblasts and glial elements. These processes induce a slight positional shift in the surrounding tissue and thereby alter the morphology of the lesioned hemisphere and result in the appearance of brain shrinkage (Shanina *et al* 2005). The infarct region on the ipsilesional hemisphere of the brain was liquefied and absorbed in most of the rats 2 weeks after focal cerebral ischemia, which is in agreement with other long-term studies (at least 14 days) using rat models of middle cerebral artery occlusion (Yanamoto *et al* 2001; Roof *et al* 2001). In a MRI study which examined the temporal evolution of ischemic lesions over time, liquefaction of necrotic tissue was observed as early as 7 days after

cerebral ischemia (Lin *et al* 2002). The liquefaction and absorption of ischemic damaged tissue might be promoted by the production of radical scavenging enzymes, higher reactive peroxynitrite and inflammatory responses triggered by oxygen free-radicals following ischemic damage (Dirnagl *et al* 1999).

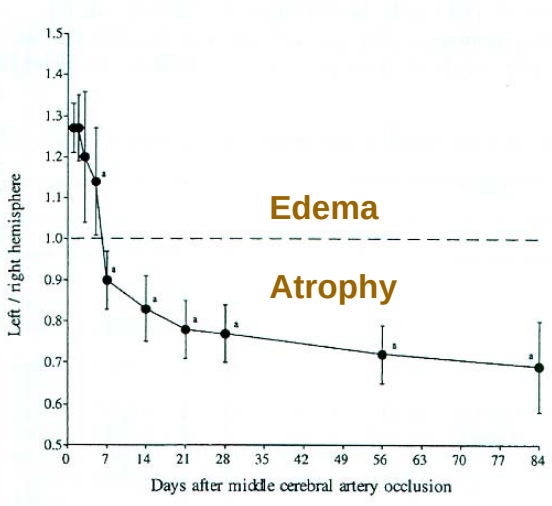


Figure 5.2 Edema index of rats with cerebral ischemia at different time points after middle cerebral artery occlusion (Takamatsu *et al* 2002)

Clinical studies have examined the changes in human skeletal muscles in hemiplegia after stroke (Dattola *et al* 1993; Hachisuka *et al* 1997). Muscle weakness of dorsiflexors such as tibialis anterior and impairment of sensory-motor control in muscles were commonly resulted after stroke. These dysfunctions led to abnormal gait pattern such as drop-foot (Burridge *et al* 2001). A few studies investigated the changes in skeletal muscles caused by lesions in central nervous system such as stroke. Abo *et al* studied the functional recovery and histomorphology of the hindlimb plantarflexors using rat with photochemically-induced stroke. It was suggested that neural reorganization in the sensorimotor cortex was the major factor responsible for the functional recovery, increase in wet weight and cross-sectional area of type I fibres of the paretic soleus muscle 3 weeks after stroke (Abo *et al*

2004). Their previous study provided evidence of neural reorganization in the contralateral non-damaged sensorimotor cortex and ipsilateral sensormotor area lateral to the induced lesion controlling the paretic hindlimb (Abo *et al* 2001). In the current study, the wet weight was a bit higher in Control group relative to the Sham group, but without significant difference. It may suggest that neural reorganization might be occurred in a lesser extent due to spontaneous recovery after stroke. There was no difference between the wet weights of tibialis anterior on both affected and unaffected side. Similar finding was reported by Choe *et al*, in which there were no differences in the hindlimb muscles weights (soleus, plantaris, gastrocnemius) between paretic and non-paretic sides 7 days after ischemic stroke. Stroke-induced undernutrition and inactivity were possible reasons for similar change in weights of the two sides (Choe *et al* 2004).

5.3 Effect of treadmill exercise on brain following cerebral ischemia

Treadmill exercise requires a repetitive locomotor performance and represents a functional activity which is purposeful bilateral hemisphere activity. Clinical studies showed that treadmill training was effective with regard to restoration of gait ability and improvement of gross motor efficiency (Smith *et al* 1999; Harris *et al* 2001). As mentioned previously, spontaneous recovery in neurological tests was observed in Control group while exercise might facilitate this process and led to a further functional recovery.

EX group kept losing weight significantly during the first week after stroke. The weight returned to baseline at the end of the second week. Weight loss may be induced by exercise, as reported in previous study (Aravich *et al* 1994). It was also noted that the Control group gained weight earlier than EX group in our study. Treadmill exercise may delay the general physiological recovery after cerebral ischemia.

Neurological performance in EX group was significantly improved on Day 7 in both limb placing and neurological tests. Although the changes in scores did not reached significant level in the 2nd week compared with the 1st day after stroke, it showed a trend of improvement. The rate of recovery was higher in EX group compared with the Control group according the limb placing scores. However, they exhibited similar trend and pace during the first week. It may suggest that the spontaneous recovery overwhelmed the exercise-induced recovery during the 1st week. In the 2nd week of treadmill exercise intervention, EX group scored higher than the Control group in the limb placing tests. It may be due to the fact that ischemia-induced recovery had reached the plateau, as demonstrated by previous study, in which spontaneous recovery reached the peak during the 1st week after stroke and persisted to 14 days (Takasawa *et al* 2002).

There was almost 60% increase in brain weight in EX group, compared with Sham group. In addition to the spontaneous recovery following ischemic injury, physical exercise might also contribute to the increases in brain weight. Brain weight gain was believed to be resulted from exercise-induced astrocytosis, angiogenesis and neurogenesis. Previous studies demonstrated that treadmill exercise induced an

increase in blood vessel density (Ding *et al* 2004) and astrocyte proliferation (Li *et al* 2005) in adult brain areas known to undergo considerable plasticity such as cortex and striatum. Astrocytosis is responsible for glutamate uptake and releasing a variety of trophic factors, which are likely to influence neuronal survival and plasticity after brain injury (Swanson *et al* 2004). Neurogenesis, which increased in nerve cell growth, was also found to occur within dentate gyrus of the hippocampus in both normal rats and rats following cerebral ischemia in active running (Farmer *et al* 2004; Briones *et al* 2005). The effects of training on brain weight have been investigated in a few studies. Kolb and Whishaw showed consistent increase in overall brain weight in young healthy rats housing in an enriched environment and this structural change was believed to lead to brain plasticity (Kolb and Whishaw 1998).

EX group had lesser brain damage compared with Control group, but without significant difference. Reduction in brain damage in EX group after cerebral ischemia was also supported by other studies with similar protocols of interventions. A series of studies conducted by Yang *et al* demonstrated the effect of 1-week treadmill exercise (started 24 hours after stroke) followed by 1-week no training, on the infarction using rats with direct middle cerebral artery occlusion (60min). They found that the infarct volume and neurological score of the training group were significantly lower than those of the 2 week no training group (Yang *et al* 2003; Wang *et al* 2005). They also found that the infarct sizes and neurological scores diminished with time in the non-training groups, due to spontaneous recovery. In the training groups, the infarct volume was getting smaller with long time of training

durations, comparing between one-week, two-week and four-week training (Yang 2003). In our current study, the neurogenesis and angiogenesis may lead to the exercise-induced reduction in infarct size. Together with the spontaneous recovery, neuron proliferation and growth of new capillaries in the penumbral area could result in decreased infarction after focal cerebral ischemia. The larger brain damage and necrotic tissue loss in Control group may also account for lesser brain weight, compared with the EX group.

Shrinkage of the ischemic hemisphere in EX groups was less severe than the Control group, but with no significant difference. Exercise may reduce atrophy of the ipsilesional hemisphere after middle cerebral artery occlusion. Apart from the increase in cell proliferation through ischemia-induced and exercise-induced neurogenesis, the reduction in atrophy in EX group could be explained by the increased levels of neurotrophic factors triggered by treadmill exercise (Cotman and Berchtold 2002; Adlard *et al* 2004). These neurotrophic factors such as brain-derived neurotrophic factor (BDNF), nerve growth factors and fibroblast growth factor, support the survival and growth of many neuronal subtypes (Cotman and Berchtold 2002). BDNF treatment after transient forebrain ischemia prevented hippocampal neuronal death and reduced infarct size by inhibiting apoptosis in rats (Beck *et al* 1994; Schabitz *et al* 2000). Thus, the ipsilesional hemisphere had lesser overall brain tissue loss and resulted in less severe atrophy.

The muscle weight of tibialis anterior on the affected hindlimb in EX group was similar to that in Control group. No significant difference was found between affected and unaffected sides, and between EX, Control and Sham groups.

Treadmill exercise for two weeks may impose no effects on the wet weight of hindlimb muscles. This finding was supported by previous studies, in which the wet weights of soleus, plantaris and extensor digitorum longus had no different between the group with treadmill exercise and control group without exercise using healthy young rats (Maxwell *et al* 1992; Brown *et al* 2003).

5.4 Effect of neuromuscular electrical stimulation on brain following cerebral ischemia

There was no significant loss or gain of body weight in ES group throughout the two weeks. The mean change in body weight was close to that in Control during the first week, indicating that weight loss may be due to cerebral ischemia (Dittmar *et al* 2003, Choe *et al* 2004) while ES did not impose further effect. During the 2nd week, ES group kept losing weight and then return back to baseline level at the end of the 2nd week. Similar to the effect of treadmill exercise, neuromuscular electrical stimulation may delay the general physiological recovery after cerebral ischemia.

ES group showed no significant improvement in postural reflexes test and limb placing tests throughout the 2 weeks. The scores kept at a narrow range in both tests (ranges of mean score in postural reflex and limb placing test = 1.00-1.33, 2-6 respectively). The trends of the scores in ES group were similar to that of Control group during the 1st week after stroke. This may imply that ischemia-induced recovery was taking place while intervention of neuromuscular stimulation on hindlimb muscles exhibited no effects on the recovery. During the 2nd week, spontaneous recovery continued (as indicated by Control group) whereas ES group

still maintained at similar level as in the 1st week. It might suggest that electrical stimulation intervention might inhibit ischemia-induced recovery in the first two weeks after stroke. Abo and colleagues showed that the behavioral recovery might be due to the functional reorganization and neuromodulation of the brain region. Functional magnetic resonance imaging (fMRI) technique revealed the activation at non-damaged contralateral sensorimotor cortex and region located lateral to the lesion on the ipsilateral cortex during electrical stimulation was applied on the paretic hindlimb (Abo *et al* 2001). Similar activations in primary sensorimotor cortex were noted during neuromuscular electrical stimulation in previous clinical studies (For details, refer to Section 2.4.2). It was suggested that the activations implied plastic reorganization of the brain. However, in the current study, no functional improvement was observed with 2-week intervention of neuromuscular electrical stimulation. The correlations between brain activations in fMRI studies, functional recovery and plastic reorganization are still unclear.

Brain weight of ES group was similar to that of Control. Apart from spontaneous recovery, there seemed to have no further physiological changes which leading to increase in brain weight, after 2-week intervention of neuromuscular electrical stimulation. Brain damage in ES group was nearly two-fold smaller than that in Control group, but with no significant difference in post-hoc analysis. Atrophy in the ipsilesional hemisphere was observed in ES group, with lesser extent (greater edema index) compared with Control group (no significant difference). The effects of neuromuscular electrical stimulation on the brain in both healthy and stroke subjects were usually studied using advanced brain imaging techniques such as

fMRI (refer to Section 2.4.2 for details). There are very limited knowledge in the literatures regarding the physiological changes in the brain induced by electrical stimulation on peripherals. A recent study showed that the electrical stimulation on median nerve was associated to decreased apoptotic neuronal injury in hippocampus using a rat model of global cerebral ischemia. It was suggested that the protective effects may be due to vascular mechanisms, in which the increase in blood flow primarily targeted the areas of the somatosensory pathway. Another possible mechanism proposed by the author based on an increase in parahippocampal inhibition, which led to decreased excitation of hippocampal pyramidal neurons (Buitrago *et al* 2004). Reduction of apoptosis in hippocampus induced by the intervention of electrical stimulation might account for the small infarct size and less atrophied hemisphere in ES group.

In ES group, muscle weight of the affected tibialis anterior was significantly higher than that in Control and Sham groups. Similar findings were found in literature that the wet weight of tibialis anterior was greater in the group with 10-week electrical stimulation after transecting the left peroneal nerve in rats, compared with the control group (Marqueste *et al* 2004). Neuromuscular electrical stimulation maintained muscle mass and strength (Dow *et al* 2004), and increased blood flow in muscle tissue (Clemente 1991). These may account for the gain in muscle weight after electrical stimulation.

5.5 Neurochemical changes caused by the interventions

Hippocampus is known to be susceptible to ischemia. Neuronal death in hippocampus under hypoxia or ischemia condition is a slow process, named delayed neuronal death which takes two to three days before presenting the final morphologic outcome. In the first two days, the cytoplasm and cellular organelles were well maintained. After 48 hours, clumping of nuclear chromatin and accumulation of non-membrane-bound dense structures were observed. On the 3rd or the 4th day, the cells were totally destroyed and disappeared (Kirino 2000). As described by Butler *et al*, only a few degenerating neurons were found in hippocampus (CA1 region) at day 1 after cerebral ischemia. There was a gradual increase in number of delayed neuronal death until day 4 (Butler *et al* 2002). The delayed neuronal death offers hope for intervention since some injured cells may be rescued by suitable treatments. In the present study, rehabilitative interventions were prescribed starting from the 1st day after cerebral ischemia. The varying levels of all the neurotransmitters in hippocampus during the 2 weeks after stroke might illustrate progressive changes of the neurons under ischemic insults.

One of the possible mechanisms for delayed neuronal death is excitotoxicity due to excessive glutamate. The excessive release of glutamate triggered a cascade of cellular and molecular events, which led to persistent impairment of protein synthesis and delayed neuronal death in hippocampal CA1 neurons (Gemba *et al* 1992). This hypothesis was supported by intracerebral microdialysis studies on animals during cerebral ischemia, in which the glutamate level was increased dramatically during ischemia (Benveniste 1991; Gemba *et al* 1992). Neuronal death

by excitoticity of glutamate was suggested to be related to the exacerbation of infarction caused by early interventions involving physical activities after cerebral ischemia (Risedal *et al* 1999). After focal cerebral ischemia, cortical networks adjacent to the infarct were hyperexcitable because of an imbalance between excitatory and inhibitory synaptic function. This imbalance was resulted from increased N-methyl-D-aspartate (NMDA) receptor-mediated excitation (NMDA receptor is one of the ionotropic glutamate receptors (Arundine and Tymianski 2004)) and decreased efficacy of GABAergic inhibition. The hyperexcitability of the surrounding tissue in the early postischemic period makes the surrounding neurons vulnerable to excitation (Risedal *et al* 1999). Presence of excitatory and toxic substances from the ischemic tissue together with the additional release of excitatory substances induced by motor activity was hypothesized to be harmful in early postischemic stage. However, the glutamate levels usually measured within one day and only the changes over time were addressed. For example, the glutamate level in striatum was found to be increased during treadmill exercise and then returned to the baseline level afterwards (Meeusen *et al* 1997). The neurochemical changes in a long-term training after stroke had not been well studied. In our study, the changing levels of neurotransmitters with days (Day 1, 2, 4, 7 and 14 after stroke) were analyzed. The results showed that both treadmill exercise and neuromuscular electrical stimulation on the contralesional hindlimb triggered the glutamate release on Day 4 and Day 7, but with no significant difference when compared with the baseline. Due to the hippocampal pyramidal cell death, the glutamate uptake system after ischemia was impaired. In Bruhn *et al*‘s study, reduced uptake of glutamate in

the extracellular space by neurons could still be observed on Day 4 after cerebral ischemia. Astrocytes become the major contributors to the clearance of excessive extracellular glutamate later on (Bruhn *et al* 2001). This might explain the decrease of glutamate levels of EX and ES groups after 4th and 7th day respectively. Our study also demonstrated, instead of exacerbation of the brain damage, the reduction of infarct size was observed in EX and ES groups compared relative to that of Control group (but without significant difference). The small increase in hippocampal glutamate during intervention (as shown in our results) or throughout the two weeks may not be sufficient to exacerbate the brain damage. Humm *et al* found that the release of glutamate triggered by overuse of impaired limbs in rats was not high enough to cause excitotoxicity (Humm *et al* 1999). According to Jiang *et al*, considerable neuronal death (over 50% loss) would be caused if the concentration of glutamate was higher than approximately 40μM, as shown in Figure 5.2 (Jiang *et al* 2005). The highest level of glutamate obtained in our study was only 3.865 μM.

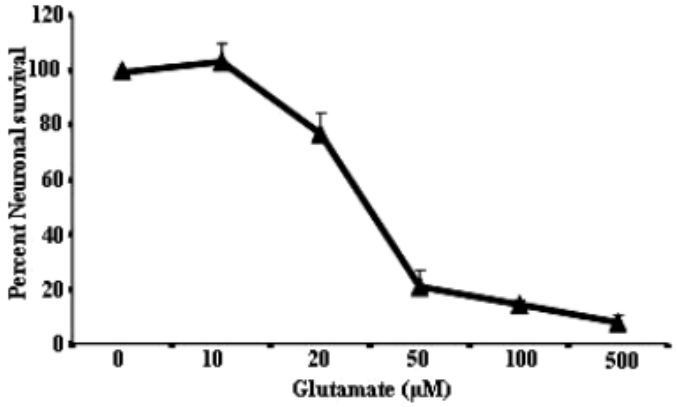
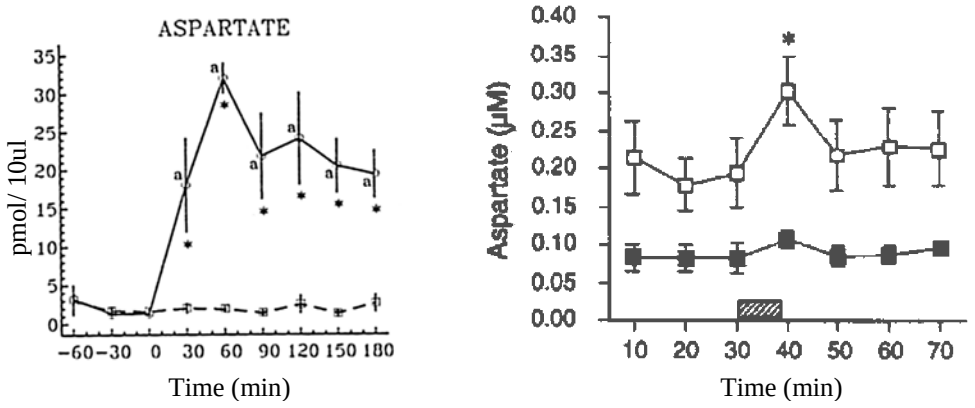


Figure 5.3 Glutamate is excitotoxic to hippocampal neurons in a concentration-dependent manner (Jiang *et al* 2005)

Aspartate is an excitatory amino acid, with similar actions as glutamate. It is neurotoxic and causes neuronal death via the same pathway as the glutamate does

(Dingledine *et al* 1999). No aspartate differences between days and group were found in our study. Similar to the glutamate, the aspartate levels reached the peak during the 1st week in the EX and ES groups. However, the aspartate level in Control group fluctuated and reached the peak on Day 4. The trends and levels of aspartate in Control and EX groups were similar, indicating that EX may not have effects on aspartate release. Aspartate level in several brain regions such as hippocampus and striatum was found to be triggered by focal cerebral ischemia and physical activity (Hillered *et al* 1989; Bland *et al* 1999). However, it seemed that these events may lead to instant elevation of the aspartate level (increased only during ischemia and during activity, as shown in Figure 5.3), instead of long-term effects on the aspartate release.



(a) (b)

Figure 5.4 The time courses of aspartate levels (a) measured in striatum during cerebral ischemia (Hillered 1989); (b) measured in hippocampus during physical activity (Bland *et al* 1999)

Taurine, an abundant inhibitory amino acid in central nervous system, plays an important role in cerebral ischemia. It has neuroinhibitory properties and might also have potential for neuroprotection in cerebral ischemia (Shuaib 2003). In our study, hippocampal taurine level of Control group kept at a high concentration during the

2-week intervention, compared with that of the EX and ES groups. Probably, the physical interventions after stroke suppressed or imposed no effects on the hippocampal taurine level. The changes of taurine levels with time in all groups (EX, ES and Control groups) on Day 4 after stroke were further analyzed. Control group still kept a significantly higher taurine level than the EX and ES group. There was an increase in the level during intervention in EX group while the level kept steady in ES group. It seemed that the taurine level showed no response to the electrical stimulation intervention. There were very few studies focused on the effects of exercise or motor activity on the brain taurine level. Previous study by Bland *et al* showed that taurine increased significantly in hippocampus in healthy rats after one week of movement stress. The authors suggested that the neurochemical responses associated with movement could involve a complicated interplay of excitatory and inhibitory neural activity (Bland *et al* 1999). Spontaneous recovery was observed based on the results of brain weight, body weight and the scores in postural reflex tests and limb placing tests in Control group. High taurine level in Control group might inhibit the influx of extracellular calcium through the reverse mode of Na^+-Ca^{2+} exchanger, which could inhibited the related calcium channels or reduced glutamate-induced membrane depolarization and thus led to excitotoxic damage (Benveniste 1991). Therefore, taurine might play an important role in the spontaneous recovery after stroke. Our results had showed that the extents of brain damage in EX and ES groups were smaller than Control group. Thus, it might indicate that taurine might not be the key factor in the reduction of brain damage in EX and ES group after ischemic insults. Further investigation on how exercise

affects the taurine level released by ischemic-injured brain cells is necessary to explain the present results.

GABA is an inhibitory amino acid and a potential neuroprotective agent. Studies showed that potentiation of GABA reduced neuronal injury. GABA inhibits glutamate release via pre-synaptic receptors (Hutchinson *et al* 2002). It increases influx of chloride ions into the cell causing hyperpolarization of the cell membrane. Then it leads to reduced excitability of the cells preventing intracellular buildup of calcium (Ravindran *et al* 1994). Continuous increase in hippocampal GABA extracellular levels throughout the first two to three days after stroke was important for neuron survival and thus attenuated ischemic damage (Johansen and Diemer 1991). The GABA levels in our study showed an increasing trend from the 1st day to the 4th day after cerebral ischemia in all groups. This may not be related to the interventions since GABA level of Control group increased with similar extent as the EX and ES groups. This might imply the spontaneous recovery took place during the 1st week after ischemia and it might suggest that GABA had similar neuroprotective action as taurine. These might be involved in the mechanism of spontaneous recovery. The levels in both treatment groups fell dramatically from the 4th day to the 14th day and returned to the levels of the 1st day after ischemia. The intervention-induced reduction of GABA release was in agreement with the findings in a recent study, it illustrated the reduced GABA receptor in hippocampus after 7 days of exercise in healthy rodents. The reduction of GABA, despite its neuroprotective nature, was claimed to be beneficial to functional recovery (Molteni *et al* 2002) since it might elevate the expression of brain-derived neurotrophic

factors in hippocampus (Zafra *et al* 1991). This might account for the smaller infarction volume in the EX and ES group, compared with the Control group.

5.6 Comparisons of the physiological effects induced by exercise and neuromuscular electrical stimulation

There was dramatic weight loss during the 1st week in EX group whereas the loss in ES group did not reach significant level throughout the two weeks. Apparently, both groups suffered from ischemia-induced weight loss. Further decrease in weight in EX group might be induced by exhaustive treadmill exercise. The general physiological condition seemed to be better in ES group, in which intervention of electrical stimulation on affected hindlimb was less vigorous and had lesser effects on the weight change. The mean change of weight showed no significant between groups.

Spontaneous recovery was dominant during the 1st week and the results in both postural reflexes and limb placing tests were similar between the two treatment groups. During the 2nd week, EX group performed better than ES group, although no significant difference was found between groups.

Both treatment groups had greater brain weight than the Sham group, but without significant difference. The additional weight in these two groups may be due to the neurogensis and angiogenesis induced following cerebral ischemia, as discussed previously. The difference in brain weights between EX and ES group did not reach significant level. The brain weight in EX group was slightly greater than that in ES

group, which was probably due to exercise-induced nerve cell proliferation in the brain. The wet weight of tibialis anterior in ES group was higher than that in EX group, but the difference did not reach significant level. Electrical stimulation may induce muscle mass, and the wet weight of hindlimb muscles was found to be not responsive to treadmill exercise when compared between treatment groups and Control group.

Both treatment groups had lesser brain damage and atrophy compared with Control group. Among the two treatment groups, the severity of brain damage and shrinkage of ipsilesional hemisphere was lesser in ES group, when compared with EX group. ES might reduce apoptosis in hippocampus, and it seemed to be more effective in reducing brain damage and atrophy of the affected hemisphere. However, none of these differences were statistically significant.

Although no significant difference was found between EX and ES group in the parameters measured, the results demonstrated the possible differences between the effects of these two interventions. Treadmill exercise seemed to be more effective in accelerating functional recovery after stroke and increasing brain weight whereas neuromuscular electrical stimulation might be better in preventing the body weight from dropping too severely, increasing the wet weight of the affected hindlimb muscles, reducing brain infarction size and atrophy on the ipsilesional hemisphere.

5.7 Critical time for interventions after stroke

As reviewed in Section 2.1 and Section 5.5, cerebral ischemic damage was mediated by multiple mechanisms that act at different times after induction of ischemia. In order to target different mechanisms involved, the treatments of ischemic brain injury have to be prescribed within a critical period (Iadecola 1999). A critical period refers to the period after stroke during which the brain is most responsive to a particular therapy (Biernaskie *et al* 2004). Figure 5.4 showed the multifaceted treatments at different critical time after stroke insults (Iadecola 1999). Within hours after ischemic insults, pharmaceutical approaches would be adopted. Drugs would be prescribed for re-establishing flow to the ischemic territory and they are most effective shortly after induction of ischemia. Medications will then be provided, which counteract the events initiating the ischemic cascade and block the processes that lead to the post-ischemic expansion of the infarct. Recovery of function aimed at enhancing the mechanisms of plasticity and repair after injury. It might be achieved by administering drugs and physical exercise.

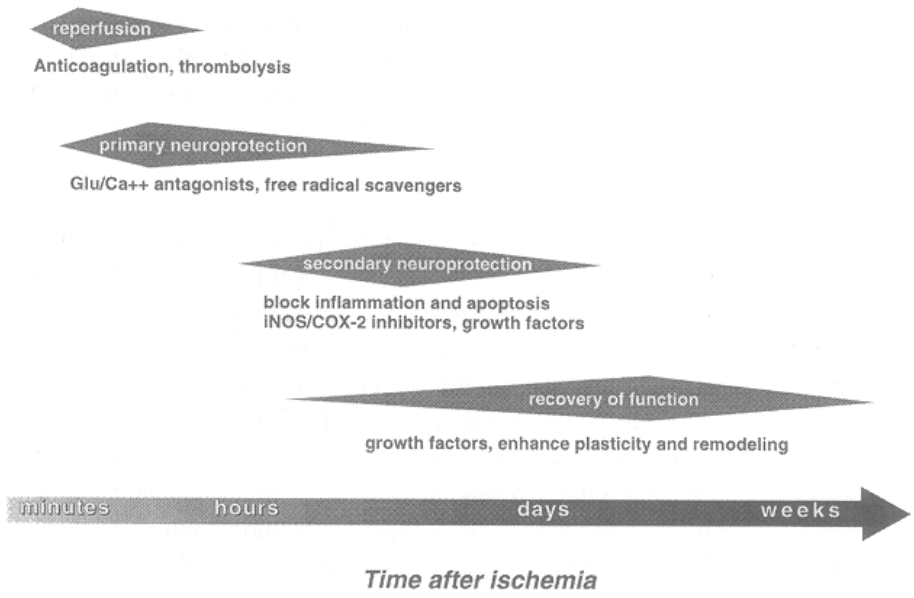


Figure 5.5 Different treatment strategies for cerebral ischemia at different time (Iadecola 1999)

During the first week, all groups (Control, EX and ES groups) exhibited similar trends and extent of recovery in the present study. The improvement observed in Control group was, most probably, due to spontaneous recovery following cerebral ischemia. The similar trends may imply the considerable contribution of spontaneous recovery to the improvement on the general physiological conditions and performance in both EX and ES groups. Regarding the neurochemical changes, GABA and taurine levels in hippocampus increased from Day 1 to Day 4 after stroke. The inhibitory actions of these two neurotransmitters were found to be neuroprotective in cerebral ischemia (refer to Section 5.5 for details). Thus, after focal cerebral ischemia, different events to protect the neurons from ischemic damage were triggered during the first week. Interventions too early after stroke may not facilitate recovery in rat models. Moreover, studies reported an exacerbation of injury and worsened overall outcome after excessive use of the

impaired limbs began immediately after the injury (Kozlowski *et al* 1996; Risedal *et al* 1999). It could be explained by the excessive sensorimotor activation too early after insult might exacerbate injury of hyperexcitable tissue surrounding the infarct (Neumann-Haefelin and Witte 2000). In order to deduct the intervention sessions which may not facilitate recovery as shown in our findings regarding the spontaneous recovery in the first week, and to avoid the risk of early intervention-induced exacerbation of brain injury, it was proposed that rehabilitative intervention should begin three to five days after insult. This allowed the hyperexcitatory status of the intact tissue around the infarct to be dissipated. This claim was supported by other studies demonstrating that rehabilitation initiated three to five days after stroke rather than in the later stage (e.g. 30 days) provided significantly greater functional compensation-recovery and enhanced structural plasticity within undamaged motor cortex in rat model of middle cerebral artery occlusion (Biernaskie *et al* 2004, Jones *et al* 1999).

5.8 Possible mechanisms of motor recovery

Plasticity refers to changes in brain networks that carry behavioral implications over time. It is an important key to motor recovery after focal cerebral ischemia (Ward 2004). One of the ways to trigger this kind of adaptive changes in injured brain was to increase in somatosensory input from the paretic hand, which might improve motor function (Conforto *et al* 2002). Treadmill exercise and neuromuscular electrical stimulation might be able to achieve this objective. Treadmill exercise is a

repetitive active movement while neuromuscular electrical stimulation is a combination of cyclic electrical stimulation on the muscles and a repetitive passive range of motion exercise (Han *et al* 2003). The two interventions showed different effects on the functional recovery, brain weight, body weight, muscle weight, brain infarction and atrophy in our study. It was believed that they might undergo different mechanisms which lead to recovery in different aspects.

As discussed in Section 5.4, our results showed a smaller infarction volume and atrophy in ES group (but with no significant differences with the Control group) and it might be due to that electrical stimulation on peripherals decreased apoptotic neuronal injury in hippocampus, as suggested by previous study (Buitrago *et al* 2004). The underlying mechanism on how such electrical stimulation suppressed apoptosis in hippocampus is not known. Apart from reducing apoptosis, neuromuscular electrical stimulation on paretic hindlimb was noted to activate the ipsilateral and contralateral hemispheres in rat with induced stroke (Abo *et al* 2001). The pathways of activation on the brain stimulated from hindlimb were shown in Figure 5.5. Abo *et al* suggested that sensory input ran contralateral via thalamus to the sensory cortex with the ipsilateral projection suppressed under normal condition (Figure 5.5a). After complete stroke at sensorimotor cortex on the right side, paresis of left hindlimb was observed (Figure 5.5b). When recovery, either spontaneous or intervention-induced, was established, the ipsilateral projection is dominating and responsible for the functional recovery due to neuromodulation and the contralateral projection showed two activated regions. The region adjacent to the lesion may reflect peri-infarct reorganization (Figure 5.5c) whereas the other one located more

lateral to the lesion may express brain plasticity (Figure 5.5d) (Abo *et al* 2001). This study revealed that neuromuscular electrical stimulation on hindlimb muscles trigger brain activation in both hemispheres. However, the relationships between the activation and physiological or functional changes have not been well understood.

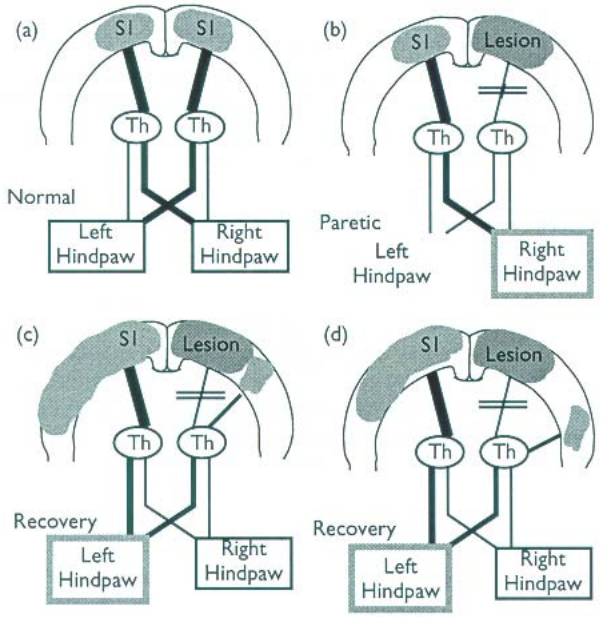


Figure 5.6 Schematic representation of activation patterns during normal conditions and after functional reorganization of the brain (Abo *et al* 2001).

Many studies had been conducted to demonstrate the benefits of physical exercise on brain tissues such as neurogenesis, angiogenesis, increase in astrocyte proliferation and induction of neurotrophic factors (refer to Section 5.3 for details). These processes may have significant contribution to the improvement in functional recovery and increasing brain weight, as shown in our results. Neurogenesis increased the number of neuron and glia in central nervous system (Johansson 2000) whereas angiogenesis increased microvascular density in cortex and striatum of rats (Li *et al* 2005). Astrocytes took an active part in synaptic plasticity. Both rapid

astrocytic changes in cortex and increased contact between astrocytes and synapses in rats in a complex environment could suggest a close relationship between astrocytic plasticity and experience-induced synaptic plasticy (Johansson 2000). Brain-derived neurotrophic factors (BDNF) are one of the neurotrophic factors commonly found in the brain. It enhanced synaptic transmission, induced long-term potentiation and stimulated synaptophysin synthesis. Mice with deficient level of BDNF showed decreased synaptic innervation and reduced levels of synaptic (Cotman and Berchtold 2002).

Ischemia-induced recovery (spontaneous recovery) and intervention-induced recovery might also undergo different mechanisms. Mechanism of motor recovery was studied based on neuroimaging and it was found that both spontaneous and intervention-induced motor recovery could involve a shift in brain activity toward more normal function. Both processes could involve compensatory changes that resulted in patterns of brain activity that diverged from normality. Neuroimaging seemed to provide limited information on the underlying mechanism. Based on the result obtained in our microdialysis study, it was noted that the glutamate level of Control group exhibited a decreasing trend while taurine and GABA level increased throughout the 2 weeks. Spontaneous recovery may depend on the increase in taruine and GABA during the 1st week after ischemia. Increase in hippocampal concentration of these two inhibitory neurotransmitters may attenuate the glutamate release and thus, protecting hippocampal neurons from excitotoxic action during ischemic injury. There is lack of information regarding the neurotransmitter levels over days after ischemic insults in the literatures. Intervention-induced recovery

seemed to be independent of the neurotransmitter levels. As discussed previously, recovery by treadmill exercise might be related to the exercise-induced events such as neurogenesis, astrocyte proliferation, and neuromuscular electrical stimulation exerted recovery effect through reducing the apoptotic neuronal death after ischemia.

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CHAPTER 6 CONCLUSIONS

In this study, the effects of treadmill exercise and neuromuscular electrical stimulation on the brain and hindlimb muscle after stroke were evaluated in different aspects. Based on our findings, the following conclusions were drawn:

1. Spontaneous recovery early after stroke was observed in Control group. It might have significant contribution to the improved performances in postural reflexes and limb placing tests in EX and ES group during the 1st week after stroke.
2. Treadmill exercise facilitated functional recovery, as shown by an increasing limb placing scores and significant improvement in neurological deficits throughout the two weeks in EX group.
3. Neuromuscular electrical stimulation significantly increased wet weight of tibialis anterior on the affected side of ES group after stroke. This may prevent the disuse atrophy of muscles after stroke.
4. Neuromuscular electrical stimulation might reduce brain damage and atrophy on the ipsilesional hemisphere. However, reduction in infarction did not result in improvement in neurological deficits.

5. In agreement with other studies, the level of glutamate and aspartate increased during intervention in EX group. However, the increases did not persist throughout the two weeks. These temporary increases of the two excitatory amino acids might not be enough to exacerbate brain damage and this might account for our findings that no exacerbation of brain damage was observed in the two treatment groups.
6. Both interventions imposed a suppression effect on taurine levels in hippocampus. This neuroprotective agent may play an important role in natural recovery in Control group, but not in treatment groups.

To the best of our knowledge, this study is the first one to demonstrate the effects of NMES on stroke recovery using the rat model of cerebral ischemia. The neurochemical findings provide an important insight into the restoration of neurotransmission induced by rehabilitation strategies (treadmill exercise and neuromuscular electrical stimulation), which may account for the motor recovery observed clinically after stroke.

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CHAPTER 7 SUUGESTIONS FOR FUTURE STUDY

Several limitations can be improved in future study, as listed below:

1. Different stimulation parameters such as frequency, intensity of the current applied, duration of the intervention, may affect the outcome measurements. It would be interesting to elucidate how these factors affect the neurological and neurochemical outcomes after stroke using rat model.
2. Physiological parameters such as heart rate, blood gas determination (pH, $PaCO_2$, PaO_2) and core temperature should be monitored before and during cerebral ischemia in rats. This is to ensure the brain damage is not due to other physiological factors e.g. acidity developed in the brain.
3. When in vivo microdialysis is used, it would be better to establish the baseline for normalizing the data, so as to eliminate the effects of individual variation on the parameter measured in the study. The baseline should be measured before inducing stroke.

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Appendix I

Form 2

Licence to Conduct Experiments APPENDIX I

Name : LEUNG Lai-yee

Address : The Hong Kong Polytechnic University,
Hunghom, Kowloon

By virtue of section 7 of the Animals (Control of Experiments) Ordinance, Chapter 340, the above-named is hereby licensed to conduct the type of experiment(s), at the place(s) and upon the conditions, hereinafter mentioned.

Type of experiment(s)

The aim of the experiment is to evaluate the efficacy of functional electrical stimulation and treadmill training on post-stroke rehabilitation in neurological, functional and physiological aspects. Stroke will be induced in Sprague-Dawley rats by middle cerebral artery occlusion under anaesthesia (by Chloral hydrate). They will be prescribed trainings after stroke and functional assessments will be conducted. All rats will be allowed to receive water and food ad libitum. At the end of the experiment, all animals will be sacrificed by cardiac perfusion of saline under deep anesthesia. Brain, muscles and bones will be removed from sacrificed animals for analysis.

Place(s) where experiment(s) may be conducted

The Hong Kong Polytechnic University

Conditions

1. Such experiments may be conducted only for research investigation.

2. The validity of this licence is from 17.3.2004 to 16.3.2006.

Dated 17 March 2004

(Dr. S.Y. LEE)
for Director of Health
Licensing Authority

Appendix IIa: Ethics Approval

THE HONG KONG
POLYTECHNIC UNIVERSITY
香港理工大學

APPENDIX IIb

MEMO

To : Dr Tong Kai-yu, Jocky Club Rehabilitation Engineering Centre

From : Dr Maureen Boost, Chairman, Animal Subjects Ethics Sub-committee

Ref. : _____ Your Ref. : _____

Tel. No. : Ext. 6391 Date : 1 0 NOV 2004

Ethical approval granted for teaching / research projects using animals
[Evaluation of post-stroke rehabilitation using electrical stimulation and gait training in a rat model of focal cerebral ischemia]
(ASESC No. 04/8)

I am pleased to inform you that approval has been given to application for change in protocol for the above project on condition that special care should be given to animals when they showed signs of infection. You will be invited to advise on the status of your project by the end of the approval validity period.

You are required to inform the Animal Subjects Ethics Sub-committee if at any time the conditions under which the animals are kept and cared for no longer fully meet the requirements of the Procedures for the Care of Laboratory Animals. If you are keeping animals in the University's animal holding room, you should state the full title of the approved project and the ASESC no. on the cage cards of the cages holding the animals. The members of the Sub-committee may visit the animal holding room unannounced at any reasonable time.

I would like to draw your attention to the University requirement that holders of licences under Cap. 340 must provide the Animal Subjects Ethics Sub-committee with a copy of their licences and a copy of their annual returns to the Licensing Authority. These must be kept up to date for the duration of the above work. In this connection, you are requested to provide to Sub-committee with the updated license for the project when available.

Dr Maureen Boost
Chairman
Animal Subjects Ethics Sub-committee

c.c. Chairman, DRC (REC)

Appendix IIb: Ethics Approval

THE HONG KONG POLYTECHNIC UNIVERSITY
香港理工大學

APPENDIX IIa

MEMO

To : Dr Tong Kai-yu, Jockey Club Rehabilitation Engineering Centre

From : Dr Maureen Boost, Chairman, Animal Subjects Ethics Sub-committee

Ref. : _____ Your Ref. : _____

Tel. No. : Ext. 6391 Date : - 3 [illegible] 2004

Application for Ethical Review for the Use of Animals in Teaching or Research
[Evaluation of post-stroke rehabilitation using electrical stimulation and gait training in a rat model of focal cerebral ischemia]
(ASESC No. 04/8)

Your application for ethics review for the use of animals in the above project has been approved for a period of two years from the date of this memo.

You are required to inform the Animal Subjects Ethics Sub-committee if at any time the conditions under which the animals are kept and cared for no longer fully meet the requirements of the Procedures for the Care of Laboratory Animals. If you are keeping animals in the University's animal holding room, you should state the full title of the approved project and the ASESC no. on the cage cards of the cages holding the animals. The members of the Sub-committee may visit the animal holding room unannounced at any reasonable time.

I would like to draw your attention to the University requirement that holders of licences under Cap. 340 must provide the Animal Subjects Ethics Sub-committee with a copy of their licences and a copy of their annual returns to the Licensing Authority. These must be kept up to date for the duration of the above work.

Dr Maureen Boost
Chairman
Animal Subjects Ethics Sub-committee

c.c. Chairman, DRC (REC)

Appendix III

Stimulation pattern based on findings from Gillis and Biewener's study on rat gait. (Gillis GB, Biewener AA, 2001)

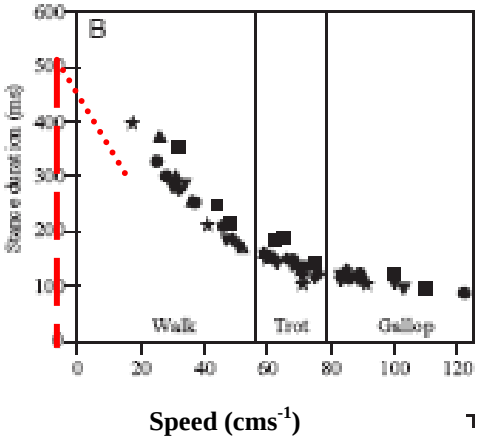

Figure A1 Relationship between stance duration and speed of walking in rat

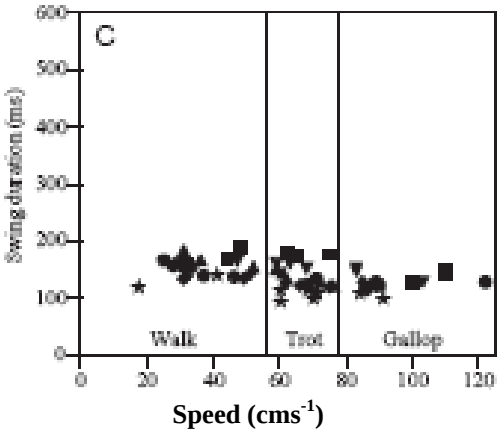

Figure A2 Relationship between swing duration and speed of walking in rat

The stance and swing durations were 400ms and 100ms respectively at the walking speed 20cm/s. The speed of treadmill in our research study (5m/min) was slower than this walking speed. Thus, according to Figure A1 with the extrapolation (dotted line), the stance phase was approximately 500ms at speed 5m/min. As shown in Figure A2, the stance duration was around 100-150ms at a wide range of speed. Therefore, the stimulation pattern in our study was designed as 150ms stimulation-on during swing phase and 500ms stimulation-off during stance phase while the walking speed (5m/min) was maintained by the treadmill. However, the stimulation pattern and the gait might not be synchronized in some rats since these rats sometimes did not walk on the running belt of the treadmill.