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EFFECTS OF DIFFERENT EXERCISES ON NEUROCHEMICAL AND FUNCTIONAL CHANGES AFTER BRAIN ISCHEMIA: AN EXPERIMENTAL STUDY

KE ZHENG

Ph.D

The Hong Kong Polytechnic University

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The Hong Kong Polytechnic University

Interdisciplinary Division of Biomedical Engineering

Effects of Different Exercises on Neurochemical and Functional Changes after Brain Ischemia: An Experimental Study

KE ZHENG

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KE Zheng

Nov 2011

Abstract

Strokes remain one of the major causes of mortality and long term disability throughout the world. Exercise paradigms have been effective rehabilitation tools in facilitating motor function recovery after strokes. Challenges in stroke rehabilitation with exercise interventions include the time window for training, the selection of different types of exercise and the mechanisms of exercise-induced recovery. Particularly, the effects of different exercise paradigms are still largely unexplored and not systematically compared. In this study, neurological deficits and cerebrovascular changes along the stroke-recovery process are investigated; the effectiveness of the voluntary, involuntary, and forced exercises in stroke rehabilitation are compared; and the possible mechanisms of the exercise-induced recovery are proposed.

100 adult Sprauge-Dowley (SD) rats were used in a pilot study to compare the ischemic stroke and hemorrhagic stroke models using ultrasonography technique and the ischemic rat model was set up after comparison. The effectiveness of different exercise interventions in stroke rehabilitation was then investigated. 150 SD rats were used at this stage. After accommodation, 33 rats were dropped-out and the remaining 117 rats were randomly distributed into four groups: Control (Con), Voluntary exercise of wheel running (V-Ex), Forced exercise of treadmill running (F-Ex), and Involuntary exercise of functional electrical stimulation (I-Ex). Ischemic strokes were induced in all group rats and 57 survival rats had motor deficits after the stroke surgery. The behavioral test and the beam walking test were conducted daily during the 7-day intervention as evaluation tools of motor recovery. Serum corticosterone and brain-derived neurotrophic factor

(BDNF) levels in the hippocampus, striatum, and cortex were measured after the rats were sacrificed. The results showed that the V-Ex group had significantly higher score in the behavioral test than all the other groups and significantly higher hippocampal BDNF concentration than the F-Ex and Con groups. On the other hand, the F-Ex group had significantly higher serum corticosterone level than the other groups. The significance of this study showed that the voluntary exercise was the most effective intervention in upregulating the hippocampal BDNF level, facilitating motor recovery, and suppressing the stress response. The results also suggested that the forced exercise was the least preferred intervention with high stress, low brain BDNF levels and less motor recovery.

PULICATIONS & AWARDS ARISING FROM THE THESIS

Publications

1. **Ke Z**, Yip SP, Li L, Zheng XX, and Tong KY. The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: a rat brain ischemia model. PLoS One. (*IF: 4.351*). 6(2):e16643 (2011).
2. **Ke Z**, Yip SP, Li L, Zheng XX, and Tong KY. The effects of voluntary, involuntary and forced exercises on motor recovery in a stroke rat model. Oral presentation. 33rd Annual International Conference of the IEEE Engineering in Medicine and Biology Society. Boston, USA. Aug 30-Sep 3 (2011).
3. Li Le, **Ke Z**, Tong KY, and Ying M. Evaluation of cerebral blood flow changes in focal cerebral ischemia rats by using transcranial Doppler ultrasonography. Ultrasound in Med & Biol. (*IF: 2.395*). 36: 595-603 (2010).
4. **Ke Z**, Kai-Yu Tong, Le Li, Xiao-Qiong Dong, Xiao-Xiang Zheng, Shea Ping Yip. Neurological and Behavioral Effects in Treadmill and Wheel exercises on Stroke Rats. Oral presentation. The 4th WACBE World Congress on Bioengineering. Hong Kong. Jul 26-29 (2009).
5. **Zheng Ke**, Michael Ying, Le Li, Shao Jie Zhang, Kai Yu Tong. Evaluation of Cerebral Blood Flow Changes in Intracerebral Hemorrhage Rats Using Transcranial Doppler Ultrasonography. (under submission)

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TABLE OF CONTENTS **Page**

List of Abbreviations

ACA	Anterior cerebral artery
ACTH	Adrenocorticotropic hormone
ANOVA	One-way analysis of variance
BA	Basilar artery
BDNF	Brain-derived neurotrophic factor
BFV	Blood flow velocity
BPM	Beat per minute
CBF	Cerebral blood flow
CCA	Common carotid artery
CNS	Central nervous system
Con	Control
CT	Computed tomography
DWI	Diffusion-weighted imaging
EE	Environmental enrichment
Edv	End distolic velocity
Et-1	Endothelin-1
ELISA	Enzyme-linked immunosorbent assay
FES	Functional electrical stimulation
F-Ex	Forced exercise
H&E	Hematoxylin-eosin
ICA	Internal carotid artery
ICC	Intraclass correlation coefficient
ICH	Intracerebral hemorrhage
I-Ex	Involuntary exercise
IP	Ischemic penumbra
LTP	Long-term potentiation
MANCOVA	Multivariate analysis of covariance
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
MCAo/r	Middle cerebral artery occlusion/reperfusion
MG	Medial gastrocnemius
MRI	Magnetic resonance imaging
PCA	Posterior cerebral artery
PRF	Pulsed repetition frequency
PWI	Persion-weighted imaging

SD	Sprague-Dawley
SD	Standard deviation
SPECT	Single photon emission computed tomography
SWD	Spontaneous waves of depolarization
TA	Tibialis anterior
TCCS	Transcranial color-coded sonography
TCD	Transcranial Doppler
TTC	Triphenyltetrazolium chloride
VEGF	Vascular endothelial growth factor
V-Ex	Voluntary exercise
Vmn	Time-averaged maximum blood flow velocity
Vp	Peak systolic velocity

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION

1.1 Stroke

1.1.1 Overview of Stroke

Stroke remains one of the major causes of mortality and long term disability throughout the world (AHA, 2007). It is the second leading cause of death globally, and is even the leading cause of adult disability in both the United States and Europe (Feigin 2005). In the United States, approximately 795,000 people suffer a new or recurrent stroke each year, from which around 15% victims die within one year (AHA, 2011). In Hong Kong in 2009, more than 25,000 patients experienced a cerebral hemorrhage or infarction, among which around 3,400 patients died within a month, and the survivors were discharged with various dysfunctions and complications (Hong Kong Hospital Authority Statistical Report, 2009-2010). Moreover, in 2010, approximately 73.7 billion US dollars were spent on stroke-related medical costs and disabilities (AHA, 2011). The costs of stroke are significant because large numbers of stroke patients require long-term hospitalization, and follow-up medical care after discharge is also necessary for their lasting motor deficits.

1.1.2 Classification of Stroke

Stroke, referred to as a cerebrovascular accident, is due to a disturbance in blood

supply to the brain, and results in the rapid developing loss of brain functions. The blood supply disturbance can be caused either by a clot obstructing the flow of blood to the brain, which is called an ischemic stroke, or by a blood vessel bursting and thus preventing blood flow to the brain, which is called a hemorrhagic stroke. On average, ischemic strokes account for over 80% of all stroke cases, but the mortality rate of this stroke subtype at one month is only approximately 10% (Koton et al. 2010). Hemorrhagic strokes are less than 20% of all stroke cases but typically have the devastating results of an around 50% mortality rate by one month (MacLellan et al. 2010).

Ischemic strokes occur when one or multiple cerebral arteries are occluded, which produces an immediate reduction in cerebral blood flow (CBF) to the areas of the brain supplied by the particular arteries, and causes a brain infarction. Ischemic strokes can be sub-classified into global ischemia and focal ischemia. A global cerebral ischemia entails a CBF diminution over the entire brain and it happens when there is a cardiac arrest or severe systemic hypotension. A focal ischemia, on the other hand, only influences restricted brain regions that are supplied by the occluded arteries. The most common cause of a focal ischemia is cerebral vascular atherosclerosis (Harukuni 2006). Generally, a focal ischemia is more common than a global ischemia (Iadecola 1999).

A hemorrhagic stroke results from rupture of a blood vessel in the brain, which not only disturbs the blood supply but the delicate chemical balance of the surrounding brain cells. Hemorrhagic strokes can also be categorized into two

subtypes, namely intracerebral hemorrhages (ICHs) or subarachnoid hemorrhages, depending on whether the brain parenchyma or the subarachnoid space, respectively, is involved (Vaughan and Bullock 1999). ICHs have a much higher incidence than subarachnoid hemorrhages, and the most common cause of an ICH is hypertension (Donnan et al. 2008).

1.2 Diagnosis and Rehabilitation

1.2.1 Diagnosis

Due to marked differences in the management and medication of ischemic and hemorrhagic strokes, the differentiation between stroke types is important at both acute and sub-acute stages. CT has been one of the most accurate methods of distinguishing between cerebral ischemia and hemorrhage by showing a bright white abnormality against a grey background (Thomas 2000). However, this technique is still not sensitive enough to detect ischemic infarctions in the early stages (0.5-12 hours) (Mayer et al. 2000). MRI has recently emerged for use in the stroke diagnosis because its sensitivity within 24 hours after the onset of stroke is superior (Ebrahim and Harwood 1999). The ultrasound is another widely applied technique since it provides a simple, relatively cheap and non-invasive imaging method. Transcranial color-coded sonography (TCCS) was firstly applied to the differential diagnosis of ischemic and hemorrhagic stroke at an acute stage by Becker and colleagues (Becker et al. 1993), and may complement

the clinical examination of acute stroke (Mäurer et al. 1998); however, this technique was limited by low spatial resolution, compared with CT or MRI, and a relatively high dropout rate (~20%) due to an insufficient acoustic bone window in patients (Seidel et al. 1995). With the application of TCCS, however, Seidel and coworkers found that an occlusion of the middle cerebral artery (MCA) branches was accompanied by a decrease in cerebral blood flow velocity (BFV) and suggested that the detection of cerebral BFV could be an indication of stroke subtypes (Seidel et al. 1995).

1.2.2 Exercises in Stroke Rehabilitation

Over 90% of stroke survivors suffer from various degrees of mental suppression and motor deficits. Medical treatment alone often has a limited effect on stroke recovery and is typically accompanied by side effects (Barber et al. 2001). As a result, post-stroke rehabilitation becomes essential and crucial in reducing the handicapping effects of the disease and facilitating motor recovery.

Exercise training is generally regarded as a vital part of stroke rehabilitation. In addition to the physical benefits, exercise has been shown to improve cognitive function and reduce depression (Dishman et al. 1996; Dunn et al. 2001). Generally, most research supports a correlation between participation in exercise and a reduction of depression and anxiety (Cotman and Engesser-Cesar 2002). Moreover, physical activity has been shown to enhance brain plasticity. A number of studies reported the enhancement of short-term potentiation and long-term

potentiation (LTP), a cellular model of learning, in the hippocampi of rats after running (Farmer et al. 2004).

Popular rehabilitation schemes include treadmill training, functional electrical stimulation (FES), robotic hands, among others. Each of these rehabilitation schemes has been individually demonstrated to be effective in facilitating motor recovery. Treadmill exercise was shown to improve ambulation through a faster walking speed, larger step length, and better walking capacity in stroke patients (Ada et al. 2003). Other research also found lower energy expenditure and improved gross motor efficiency in stroke patients after treadmill exercise (Macko et al. 2001). FES is sometimes regarded as a type of involuntary exercise as it stimulates affected muscle groups with a specific stimulation pattern and thus causes muscle movement (Park et al. 2003). It is shown that with electrical stimulation of the affected limb, stroke patients had better motor recovery and walking ability, compared to a placebo group (Yan et al. 2005). Robot hands are applied mainly in task-specific exercise schemes, and some studies suggest that voluntary activation of the damaged motor system was a key stimulus to motor recovery in robot-assisted training (Kahn et al. 2006).

1.3 Scope and Objectives

1.3.1 Rationale and Scope

There is an enormous burden of stroke in many countries around the world,

which has produced intense interest in studying effective rehabilitation training programs. Clinical studies, however, typically encounter difficulties such as limited sample size and variations among patients: the severity, infarction area and location of ischemia or hemorrhage can differ from person to person. In addition, it is very difficult to study the neurochemical changes in patients' brains, which may be a key factor in the recovery process. As a result, rehabilitation studies using animal stroke models become essential and prevalent.

Various studies have substantiated the beneficial effects of exercise on stroke recovery in animal models (DeBow et al. 2003; Marin et al. 2003; Yang et al. 2003a; Burnett et al. 2006). Among the various exercise paradigms, forced treadmill running, voluntary wheel running, and involuntary muscle movement caused by electrical stimulation are the most commonly adopted exercise models. In addition to substantiating their physical benefits, studies of these exercises also aimed to explore the post-training neurological changes, such as the regulation of brain-derived neurotrophic factor (BDNF), a leading factor in learning and memory after brain ischemia (Noble et al. 1999).

Treadmill running rats showed either a smaller brain infarct volume or better neurological function when they were trained before or after a stroke, respectively, when compared with spontaneously recovered rats (Hayes et al. 2008; Yang et al. 2003a; Ang et al. 2003). Some studies, however, suggested that beneficial effects, which expressed as an up-regulation of brain BDNF levels, existed only in low-intensity treadmill-running (15 m/min) rats; moderate-intensity treadmill

running (25 m/min) would elevate serum corticosterone levels (Soya et al. 2007). Corticosterone up-regulation is a common sign of chronic stress that typically causes reduced body weight and spleen atrophy (Brown et al. 2007), indicating a response of negative adaptation to stress. Moreover, corticosterone was shown to reduce BDNF availability in the rat hippocampus (Schaaf et al. 1998).

Wheel running is generally considered to be a type of voluntary exercise in animal models, and it does not activate a systemic stress response (Yanagita et al. 2007). Although some research suggests that voluntary wheel running is not efficient at reducing brain infarct volume compared to forced treadmill running when performed before stroke (Hayes et al. 2008), Marin et al. concluded that there was no direct relationship between brain infarct volume and motor recovery (Marin et al. 2003). Moreover, other studies found that voluntary exercise showed superior effects in terms of plastic changes in the dentate gyrus of the hippocampus (Arida et al. 2004; Collins et al. 2009). In accordance with these results, Huang and his colleagues showed that up-regulation of BDNF lasted seven and two days in wheel and treadmill groups, respectively (Huang et al. 2006).

Functional electrical stimulation (FES) is regarded as a type of involuntary exercise because it helps generate muscle contractions by stimulating the paralyzed muscle with specific stimulation patterns (Burnett et al. 2006). In animal studies, rats receiving such electrical stimulation during brain ischemia showed decreased infarct volume and better behavioral outcomes than the control group rats (Burnett et al. 2006; Leung et al. 2007). FES was also shown to up-regulate

BDNF expression in stimulated muscles in rat models (Park et al. 2003).

Although numerous studies have investigated the effects of different exercise schemes as stroke rehabilitation tools, their results may not be comparable because the experiments were conducted under different conditions. Therefore, it is still unclear that which exercise is the most effective at facilitating motor recovery and regulating neuron growth in the brain. Various studies are initiated for this question, and the answer will undoubtedly expedite progress of stroke rehabilitation.

1.3.2 Objectives

This study aims to explore the functions of voluntary, involuntary, and forced exercises as rehabilitation interventions in the regulation of the stress response and brain BDNF expression after ischemic strokes. In parallel, we also compare the effects of these exercises on facilitating motor recovery after brain ischemia in a rat model. It is hypothesized that the significant differences will be found among different exercise-intervention groups. The results of the current study will enhance our understanding of the effectiveness of different rehabilitation exercises and their effectiveness and provide information for future clinical practice.

The specific objectives of the study were:

1. To setup stroke rat models and investigate the differences of cerebral blood flow changes, brain damage areas and neurological deficits between ischemic

strokes and hemorrhagic strokes;

2. To compare the motor function recovery level using De Ryck's behavioral scoring system in voluntary, involuntary, and forced exercise group rats;
3. To compare the neurochemical effects including brain BDNF level and serum corticosterone level after exercise training between the three groups; and
4. To explore the relationship between motor recovery, brain BDNF and serum corticosterone levels following stroke.

1.4 Outline of the Thesis

Following this introductory chapter, Chapter 2 begins with a review of the fundamentals of stroke pathophysiology. Different animal models of stroke are then introduced and compared. Evaluation tools and their specifications for experimental stroke are then presented. Finally, stroke rehabilitation in animal models is reviewed.

Chapter 3 presents the experimental methods for this study. Ischemic stroke model and hemorrhagic stroke model in rats are set up and investigated. The ischemic stroke model is then selected and applied to compare the effectiveness of voluntary, involuntary, and forced exercises as stroke rehabilitation interventions.

Chapter 4 shows the results of the investigation of both ischemic stroke model

and hemorrhagic stroke model, and also the comparison of the effectiveness between the three exercise rehabilitation interventions.

Chapter 5 begins with a discussion of experimental setup of both the ischemic stroke model and the hemorrhagic stroke model. After that, the possible mechanisms of neurological changes and motor recovery levels after ischemic stroke in different exercise intervention groups are revealed.

Chapter 6 summarizes the findings of this study and the potential implications regarding different exercise schemes. Possible research questions are raised and suggestions for future work are provided.

CHAPTER 2

LITERATURE REVIEW

2.1 Animal Models of Stroke

Animal models of stroke provoke pathophysiological states that are similar to those of human stroke, and studies using animal models have provided profound knowledge regarding both cerebral ischemia and hemorrhage and evidence to support the efficacy of treatment strategies and rehabilitation schemes. To imitate the human stroke mechanisms, ischemic stroke models and hemorrhagic stroke models have been developed using animal models. In ischemic stroke models, major artery/arteries are occluded to restrain the blood flow to certain brain regions, whereas in hemorrhagic stroke models, an intracerebral hemorrhage is artificially created with a collagenase or autologous blood injection. Both types of animal stroke models induce neurological impairments and present as motor deficits that can be systematically scaled with various evaluation tools.

2.1.1 Cerebral Ischemic Models

Animal cerebral ischemia models typically fall into two main topographic categories, global and focal cerebral ischemia. Global cerebral ischemia occurs when the CBF in most or all of the brain is reduced, usually due to cardiac arrest or coronary artery bypass graft surgical interventions (Campbell and Hunter 1999). This widespread reduction or cessation of blood flow causes selective

neuronal death primarily in the hippocampus and striatum. Focal cerebral ischemia refers to a decrease of CBF in a very distinct and specific region of the brain, typically the middle cerebral artery (MCA). In rats with an MCA occlusion, the greatest flow reduction was found in the striatum and the penumbra (Li and Fisher 2001). Figure 2.1 shows the main arteries supplying the brain in human (left) and rat (right). An understanding of the cerebral arterial structure may facilitate the comprehension of the different ischemic models in the following sections.

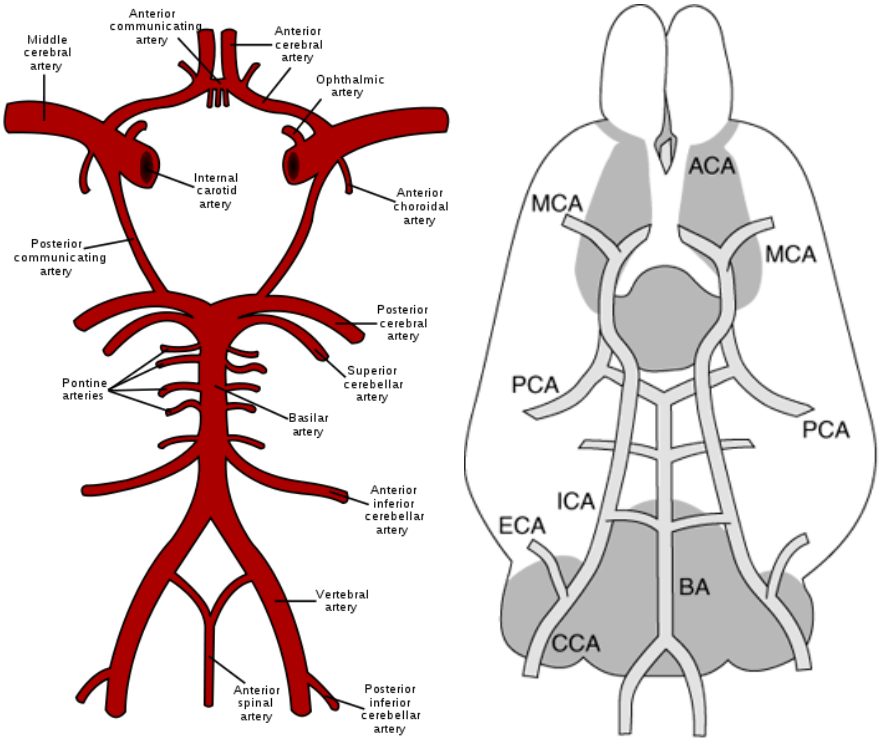


Figure 2.1. Major cerebral arterial structures in brains of humans (left) and rats (right). In both humans and rats, bilateral MCA, bilateral PCA, bilateral ICA, ACA and BA are the major arteries supplying the brain. ACA: anterior communication artery; CCA: common carotid artery; ICA: internal carotid artery; ECA: external carotid artery; MCA: middle cerebral artery; PCA: posterior cerebral artery; BA: basilar artery. (from Wikimedia Commons; O'Neill and Clemens 2001)

2.1.1.1 Global Cerebral Ischemic Model

Global cerebral ischemia entails a reduction in CBF over the entire brain, and

causes neuronal injury to selectively vulnerable brain areas. One of the simplest methods to produce global ischemia without recirculation is decapitation (Lowry et al. 1964). This method, however, does not allow any modulations. Neck tourniquets have been widely applied in rat models, but have complicating problems such as venous congestion and vagal nerve compression (Siemkowicz and Gjedde 1980). Ventricular fibrillation is another technique to produce complete global cerebral ischemia and mimics the clinical situation of cardiac arrest (Michael et al. 1984). This technique is generally used in large animals and is therefore very expensive and labor intensive. A four-vessel occlusion model was developed to provide a low-cost method of reversible forebrain cerebral ischemia in rats (Pulsinelli and Buchan 1988). In this method, briefly, bilateral vertebral arteries are electrocauterized and bilateral CCAs are occluded. Although the success rate was 50% to 75% with this model, the effects of ischemia were quite variable due to the variability of collateral circulation present in each strain of rats (Pulsinelli and Buchan 1988). A two-vessel occlusion model was developed and used as an alternate to the four-vessel occlusion model. In this model, bilateral common carotid artery occlusion is coupled with systemic hypotension to produce a reversible forebrain ischemia (Dietrich et al. 1993). The major advantages of the two-vessel occlusion model are that it requires an easier surgical preparation, a readily accomplished reperfusion, and chronic survival study applicability (Lin et al. 1992; Traystman 2003). However, anesthesia and hypotension with drugs are required, which can confound data interpretation

(Traystman 2003). In general, the global cerebral ischemic models are used for investigating biochemical, metabolic and physiologic responses after reductions in CBF.

2.1.1.2 Focal Cerebral Ischemic Models

Focal cerebral ischemia models have been widely applied in stroke-related research due to their strong resemblance to human ischemic strokes. Most focal cerebral ischemia models involve the occlusion of one major cerebral blood vessel, such as MCA, because it was suggested that up to 80% of all strokes result from ischemic damage in the MCA area (Mohr et al. 1986). MCA occlusion (MCAo) results in a reduction of CBF mainly in the striatum and cortex, and induces large infarctions in these regions. The degree and size of infarctions, however, depends on the duration of the MCAo, the site of occlusion along the MCA, the amount of collateral blood flow into the MCA territory (Traystman 2003). To control these variables, several models were designed to obtain consistent infarcts and to allow control over the time of occlusion and of the events that occur after reperfusion. These models can be categorized from permanent or temporary according to time of MCAo, and as proximal or distal with respect to the position of the MCAo. Common focal cerebral ischemia models and their categories are listed in Table 2.1

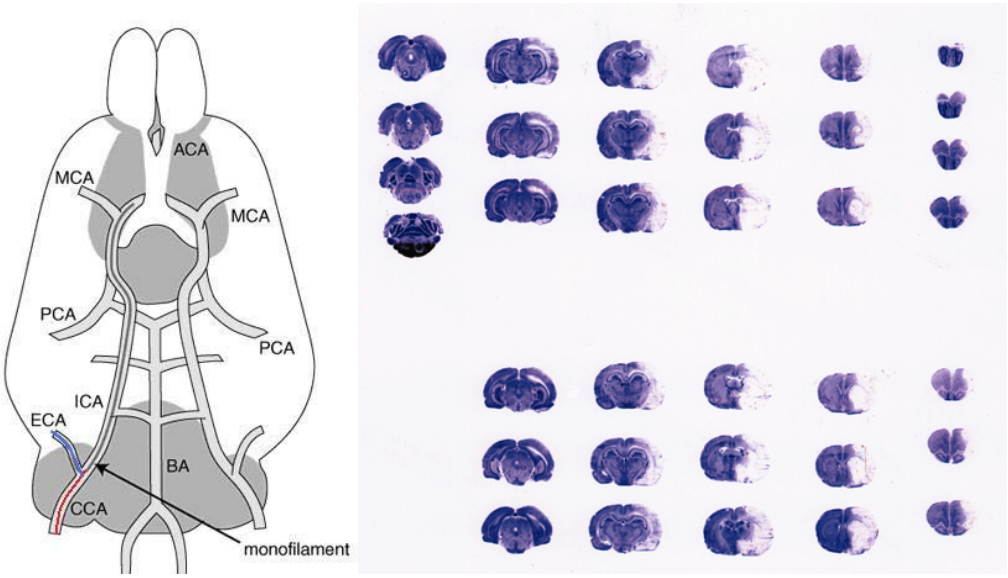
Table 2.1 Animal Models of Focal Cerebral Ischemia

Focal ischemia models	Occlusion type/Position
Intraluminal suture MCAo	Transient / Proximal
Permanent MCAo	Permanent / Proximal & Distal
Endothelin-1 (Et-1) MCAo	Transient / Proximal
Photochemical MCAo	Permanent / Distal
Blood clot embolization	Permanent/ Proximal or Distal

2.1.1.2.1 Intraluminal suture MCAo model

The intraluminal suture middle cerebral artery occlusion model was developed by Koizumi et al. in rats, and was described as a reliable, minimally invasive technique in rat focal stroke model of a temporary regional ischemia (Koizumi et al. 1986; Longa et al. 1989). This technique involves inserting a 4-0 thread from either the CCA or the ECA into ICA then advancing the thread cranially to block the MCA (Fig 2.2a). The length of the thread from origin of the ICA or the ECA is typically 17-20mm, which allows the simultaneous occlusion of collateral circulation from ACA. This technique was augmented by coating the thread with poly-L-lysine to improve the adhesion of the suture to the surrounding endothelium, in which lead to more consistent infarctions (Belayev et al. 1996). CBF was found to decrease by 80% during the occlusion and returned to a normal level after reperfusion by withdrawing the suture (Memezawa et al. 1992). Damage is seen in the striatum and frontoparietal cortex, where CBF reduction was greatest (Fig 2.2b). This intraluminal suture occlusion technique models focal infarction in a large vascular territory and does not require a craniotomy (Li and Fisher 2001). Although microsurgical skills are required, it can still be

regarded as relatively simple, and therefore is currently a valid and widely used method in various stroke studies.



(a) (b)

Figure 2.2. Intraluminal suture middle cerebral artery occlusion model (a) and brain damage map (b). (a) A 4-0 nylon thread is inserted from either the ECA (blue) or the CCA (red), and advanced to block MCA. (b) Cresyl violet staining of a rat brain 24 hours after an MCAo using the intraluminal suture model. Infarction is visible as lighter staining in the frontoparietal cortex and striatum. (from O'Neill and Clemens 2001).

2.1.1.2.2 Permanent MCAo model

A permanent MCAo model, which is also recognized as the Tamura model, was used extensively from the late 1980s until the mid 1990s and was mainly for testing neuroprotective agents (O'Neill and Clemens 2001). This model involves a subtemporal craniotomy to access the proximal regions of the MCA in rats (Fig 2.3). After exposure, the MCA is occluded proximal to the lateral lenticulostriate branches by electrocoagulation (O'Neill and Clemens 2001). This model results in a permanent infarction of the cortex and striatum.

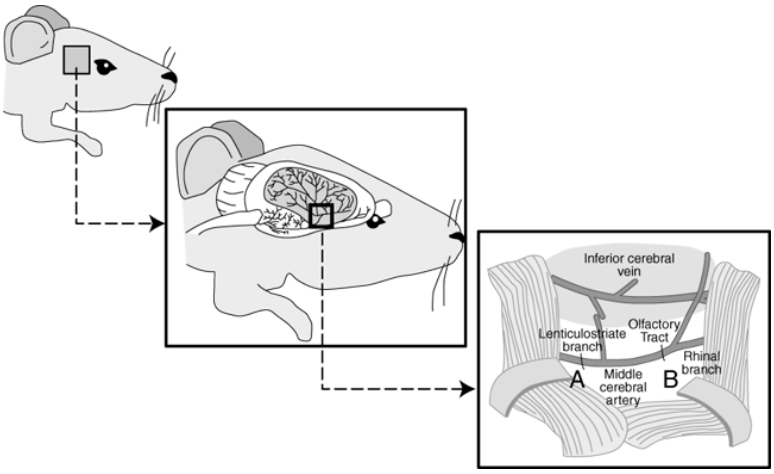


Figure 2.3. Position of the craniotomy in a permanent MCAo model. In this model, both proximal (A) and distal (B) sites of MCA are electrocoagulated to yield cortical and striatal infarcts. (from O'Neill and Clemens 2001).

2.1.1.2.3 Endothelin-1 (Et-1) MCAo model

Endothelin-1 is a potent vasoconstrictor and was firstly used in stroke rat model in 1993 (Sharkey et al. 1993). In this model, Et-1 is injected to the brain region adjacent to MCA via a previously implanted guide cannula. Occlusion of MCA is immediately but disappears gradually. As a result, this model is a fast and noninvasive method to produce MCAo but is not suitable in drug development or evaluation since the duration of ischemia is not controllable and the damage is variable (Li and Fisher 2001).

2.1.1.2.4 Photochemical MCAo model

The photochemical MCAo model involves irradiation of several branches of the distal MCA with beams from an argon dye laser following intravenous infusion of the photosensitizing dye Rose-Bengal (Watson 1997). This method generates endothelial damage to produce arterial thrombi consisting of platelets,

erythrocytes, and putative fibrin similar in structure to those of clinical stroke (Green and Cross 1997). This model requires only restricted craniotomy, but often induces unwanted microvascular injuries as well.

2.1.1.2.5 Blood clot embolization model

The blood clot embolization model is primarily used in studies that examine thrombolytic agents and the protective effects of drugs (Green and Cross 1997). In this model, homologous blood clot fragments are injected directly into the common carotid artery or into a carotid artery via a retrograde catheter placed in an external carotid artery (Zhang et al. 1997). The emboli, however, typically flow into various branches of the vessel and lead to inconsistent infarct locations. Therefore, this model is seldom used in studying ischemia mechanisms and therapeutic interventions.

2.1.1.2.6 Summery of focal ischemia models

To compare the different focal ischemia models, their advantages and disadvantages are listed in Table 2.2.

Table 2.2. Advantages, disadvantages and features of different models of focal cerebral ischemia models (Campbell and Hunter 1999; Traystman 2003)

	Advantages	**Disadvantages**
Intraluminal suture MCAo	No craniotomy; Simple surgery, allowing rapid screening of potential neuroprotectants; Reperfusion easily performed; Reliable measurement of damage Mimic the human ischemic strokes most	Occlusion status invisible; Relatively high death rate
Permanent MCAo	High successful rate	Craniotomy required; Extensive surgery required; Occlusion is permanent; High death rate
Et-1 MCAo	Minimally invasive; Applicable to rapid screening of potential neuroprotectants	Skilled manipulation required; Considerable variability in wearing off of the endothelin
Photochemical MCAo model	Small craniotomy	Microvascular injury; Blood-brain-barrier impaired; Pathology not demonstrated to be representative of thrombotic stroke in humans
Blood clot embolization model	No craniotomy	Inconsistency in infarct location and size

According to their advantages and disadvantages, intraluminal suture MCAo model has become the typical method in stroke studies using animal models.

2.1.2 Intracerebral Hemorrhage (ICH) models

Animal ICH models have been developed to reproduce the general important pathophysiological events documented in human ICHs, including edema development, markedly reduced metabolism, and tissue pathologic responses (Andaluz et al. 2002). The most commonly used models involve infusion of

either autologous blood or bacterial collagenase into the striatum of rats (Bullock et al. 1984; Rosenberg et al. 1990). Although both of these models recreate the fundamental human ICH responses, they differ in ways that influence stroke outcomes (MacLellan et al. 2010).

2.1.2.1 Autologous Blood Infusion Model

The autologous blood injection model was one of the earliest ICH models and was developed to mimic the single large bleed that was thought to occur in most ICH patients (Kirkman et al. 2011). In this model, blood is taken from a superficial vessel and stereotaxically injected into the brain, typically into the striatum (Bullock et al. 1984). The injections of blood, however, are often complicated by subarachnoid hemorrhages and thus produce inconsistent hematoma volumes (MacLellan et al. 2008). It was found that hematoma extensions into adjacent white matter occurred in 25% of animals (Xue and Del Bigio 2003). As a result, this model is routinely used for hemorrhage mechanisms investigations, but it is not appropriate to study bleeding processes or interventions that may affect bleeding (Xi et al. 2006).

2.1.2.2 Bacterial Collagenase Infusion Model

Injections of bacterial collagenase into the striatum of rats disrupt the basal lamina of cerebral blood vessels, causing blood leakage into the surrounding brain tissue (Clark et al. 1998; Rosenberg et al. 1990). In this method, bleeding

begins ten minutes after the injection and develops into a hematoma in 4 to 24 hours (Rosenberg et al. 1990). This simple and reproducible model is widely used because the hemorrhage occurs from *in situ* vessels and imitates the hematoma expansion of human ICH (Wang and Tsirka 2005). Researchers found this method to be simpler than blood injections without the complication of blood backflow along the needle track (Kirkman et al. 2011).

2.1.2.3 Summery of Intracerebral Hemorrhage Models

A comparison of the autologous blood injection model and the bacterial collagenase injection model is shown in Table 2.3.

Table 2.3. Comparison of the two most common ICH modeling techniques (Kirkman et al. 2011)

	Autologous blood injection	**Collagenase injection**
Research purpose	Investigating mechanisms of hemorrhagic damage	Assessing long-term functional outcomes following ICH
Advantages	Consistent hemorrhage volume; Clinically relevant comparison	Simulates human disease more accurately; Simple procedure; Dose-dependent hemorrhage size; Mimics spontaneous bleed; May imitate bleeding-rebleeding phenomenon
Disadvantages	Not a spontaneous bleed; Not suitable for evaluation of rebleeding; Does not mimic microvascular breakdown effects; Not suitable for long-term study	Neurotoxicity of collagenase

2.2 Stroke Pathophysiology

Investigation of neurochemical changes during stroke can help the understanding the mechanisms of different stroke models. Two major mechanisms causing brain damage in stroke are ischemia and hemorrhage. Ischemic strokes account for 80% of all cases, while non-traumatic intracerebral hemorrhages represent approximately 15% of all strokes. In an ischemic stroke, sharply decreased or absent circulating blood deprives neurons of necessary substrates and result in rapid tissue death due to the loss of brain anaerobic metabolism (Jones et al. 1981). Intracerebral hemorrhages originate from cerebral vessel bursts and frustrate the brain by disrupting connecting pathways and causing localized pressure injuries (Zamir and Silver 1992). In both types of strokes, destructive biochemical substances are released and devastating events follow.

The development of novel and effective therapeutic strategies for stroke requires continuous investigation and comprehensive understanding of stroke pathophysiology (Dirnag et al. 1999). In this chapter, the pathophysiology of both cerebral ischemia and non-traumatic hemorrhages is illustrated on a cellular basis, and the portrait of the processes may further the fundamental knowledge of stroke.

2.2.1 Cerebral Ischemia

Cerebral ischemia can be classed by topography as either global or focal and by chronology as either reversible or irreversible (Özben 2003). Occlusion of one of

the major cerebral arteries, typically the middle cerebral artery (MCA), produces an immediate reduction in cerebral blood flow (CBF) to the areas of the brain supplied by that particular artery. Normal CBF is approximately 50-60 ml/100 g/min in the adult human and varies in different parts of the brain. During the ischemia, reduction of a CBF to below 20 ml/100 g/min causes an electrical silence and diminished synaptic activity ensues in an attempt to preserve energy stores; a reduction of CBF below 10 ml/100 g/min leads to irreversible neuronal injury and infarction regardless of its duration (Pulsinelli 1995; Heros 1994). The reduction of CBF, however, is not homogenous throughout the ischemic territory but is greatest in the center, which is called the ischemic core. The ischemia becomes less dense away from the ischemic core until flow returns to normal in regions supplied by adjacent arteries that are not occluded. This peripheral region that lies between the normally perfused brain and the occluded territory, termed the ischemic penumbra (IP), is characterized by the preservation of some energy metabolism because the CBF in this area is 25% to 50% of normal (Astrup et al. 1981). In the penumbra, pathophysiological mechanisms are dynamic; cells in this zone may be salvaged by reperfusion or by other health care approaches (Seisj 1992). In contrast, cells in the ischemic core are usually doomed, and there are five recognized fundamental mechanisms that can lead to cell death: energy failure and ionic imbalance, oxygen radical generation, inflammation, apoptosis, and peri-infarct depolarization (Dirnag et al. 1999; Traystman 2003; Iadecola and Alexander 2001). Figure 2.4 shows the major pathways implicated in ischemic

cell death.

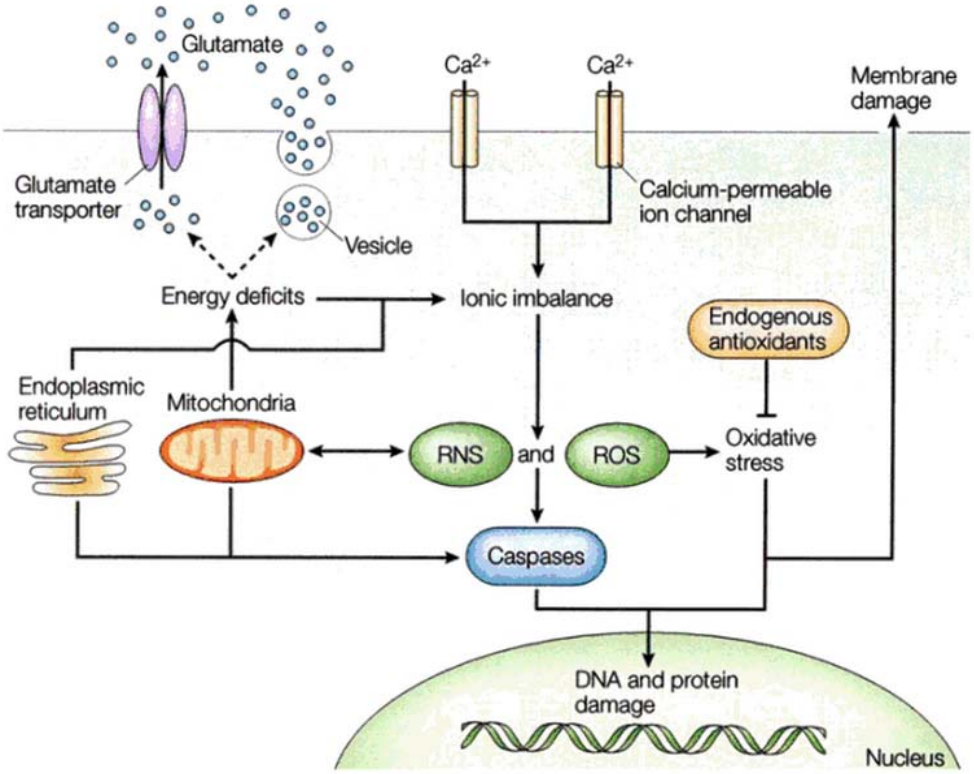


Figure 2.4. Major pathways implicated in ischemic cell death. There is extensive interaction and overlap between multiple mediators of cell injury and cell death. (from Lo et al. 2003).

2.2.1.1 Energy Failure and Ionic Imbalance

An interruption of the blood supply to the brain results in an impaired cellular energy metabolism and failure of energy-dependent processes such as the Na^+/K^+-ATPase, which further disturbs electrochemical gradients across the plasma membranes of neurons and glial cells (Stys 1998). Cell membrane depolarization occurs when Na^+ and Ca^{2+} ions accumulate in the cell and K^+ ions efflux from cells to extracellular space (Iadecola 1999). Massive amounts of transmitters are released due to this increased extracellular K^+ concentration, which triggers ionic fluxes through voltage-dependent and agonist-operated channels (Nilius and Droogmans 2001). On the other hand, increased

extracellular transmitter concentrations (particularly glutamate and aspirate) also cause the entrance of Na^+ and Cl^- into neurons and glial cells via monovalent ion channels (Pulsinelli 1992). These events induce a further increase in extracellular K^+ ions and an uptake of Na^+, Cl^+ and Ca^{2+} ions, although the influx of Na^+, Cl^+ and Ca^{2+} ions is much greater then efflux of K^+ ions. One direct consequence of such a gradient disturbance is edema, becasue (1) the cellular accumulation of ions causes the formation of cytotoxic edema; and (2) water tends to move into the cells by osmosis (Özben 2003; Kristian and Siesjo 1997). Prolonged edema may cause cell lysis and membrane injury, which eventuates in the neuronal death. Energy failure is suggested to be the predominant mechanism of cell death in the ischemic core (Astrup et al. 1977).

2.2.1.2 Oxygen Radical Generation

Ion gradient disturbances, particularly the high intracellular Ca^{2+} concentration, stimulate excessive mitochondrial oxygen free radical production in neurons and glial cells, which activates proteolytic enzymes and increases NO production by neuronal nitric oxide synthase (Chan 2001). Proteolytic enzymes degrade cytoskeletal proteins and extracellular matrix proteins, whereas NO causes cytotoxicity through the formation of iron-NO complexes with several enzymes, including the mitochondrial electron transport chain, protein sulfhydryls oxidation and DNA nitration (Zhang et al. 1994). In addition to its neurotoxic effect, NO is also considered a neuromodulator and facilitates the release or

inhibits the uptake of neurotransmitter, including dopamine, acethylcholine and glutamate, in neurons (Dalkara et al. 1997). Unfortunately, extracellular glutamate overload further causes an intracellular influx of Na^+ and Ca^{2+} and a reaction cascade, as described previously.

Apart from the high intracellular Ca^{2+} concentration, free radicals can also come from iron ions released by the intramitochondrial electron transfer chain in injured brain cells (Kroemer and Reed 2000). During ischemia, mitochondrial oxidative phosphorylation is inhibited, which decreases ATP production and enhances anaerobic metabolism. The depletion of ATP and the suppression of the oxidative metabolism lead to an insufficient amount of 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 an increase in mitochondrial free radical production. Free radicals are always a major cause of mutations, and they may also cause DNA cross link and DNA strand breaks in mitochondrial genes (Bernardi et al. 2001). Impaired mitochondrial genes hamper cell respiration, further increasing the possibility of oxygen radical production and neuronal death.

2.2.1.3 Inflammation

Inflammation is intricately related to ischemia and to subsequent tissue damage. Acute local inflammation is characterized by the infiltration of leukocytes, and involves the accumulation of polymorphonuclear neutrophils (PMN) (Kostulas et

al. 1999). Oxygen free radicals generated by abnormal intracellular calcium regulation, trigger the expression of a number of proinflammatory genes in injured cells and consequently produce inflammatory mediators such as cytokines, chemokines and adhesion molecules (Pantoni et al. 1998). The over-expression of these inflammatory mediators promotes the accumulation of leukocytes in the hypoxic area. Leukocytes, particularly PMN, have detrimental effects by exacerbating tissue damage through both the physical obstruction of vessels and the production of oxide and superoxide, proinflammatory cytokines, and cytolytic enzymes (Danton and Dietrich 2003; Wen et al. 2006). Another harmful effect of inflammatory mediators is the release of proteolytic enzymes, which may cause lysis of endothelial cell membranes and the basal lamina (Romanic et al. 1998). Damage of the endothelial cell-basal lamina lining may facilitate the blood cells loss and the hemorrhagic transformation of a brain infarct (Wen et al. 2006; Hamann et al. 1996).

Interestingly, inflammatory cascades can stimulate not only detrimental but potentially beneficial pathways after ischemia. The peptide vascular endothelial growth factor (VEGF), for example, was found to exacerbate edema in the acute phase of a cerebral ischemia but shown to promote vascular remodeling during stroke recovery (Zhang et al. 2000). Afterwards, the net effect of inflammation depends upon the stage of the brain injury in multiple divergent pathways (Fig 2.5).

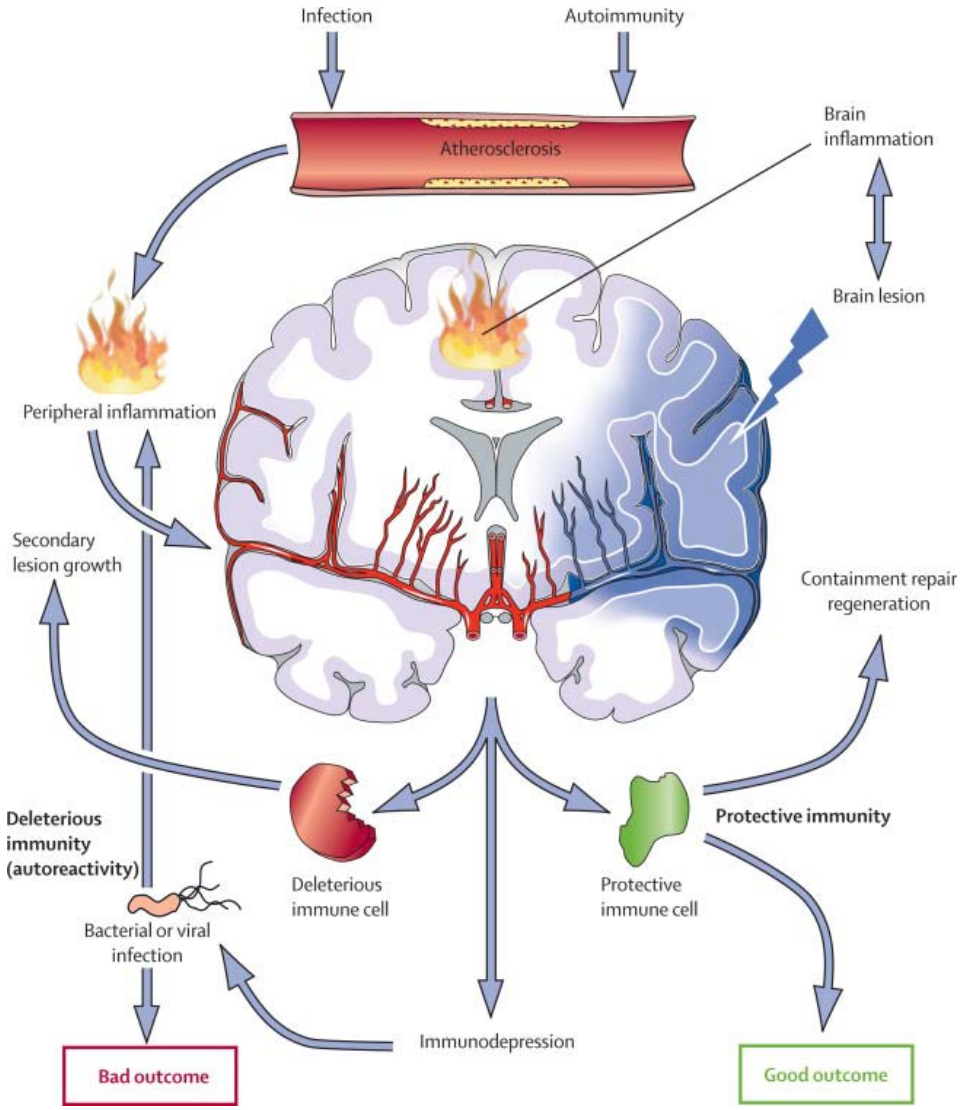


Figure 2.5. Inflammation can stimulate both detrimental and beneficial pathways after a cerebral ischemia. A complex interaction between various mediators is shown, and the net effect of inflammation depends on the level of tissue injury and the predominance of a single cascade among multiple divergent pathways. (from Macrez et al. 2011).

2.2.1.4 Apoptosis

Apoptosis is also called programmed cell death and is recognized histologically by cells positive for terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labeling that exhibits DNA laddering (Yuan and Yankner 2000). Apoptotic mechanisms begin within one hour after ischemic injury. During apoptosis, damage first occurs in the nucleus where protein-cleaving enzymes

known as caspases are released (Choi 1996; Kajstra et al. 1996). The activation of caspases triggers several inter-related events, including the release of oxygen free radicals, death receptor ligation, DNA cleavage, and lysosomal protease activation (Budd et al. 2000; Salvesen 2001). These events ultimately destroy the integrity of the mitochondrial membrane and plasma and cause cell death. Because caspases-dependent cell death requires energy in the form of ATP, apoptosis predominantly occurs in the IP rather than in the ischemic core, where there is complete energy failure (Nicotera et al. 2000).

2.2.1.5 Peri-infarct Depolarization

A focal cerebral ischemia induces spontaneous waves of depolarization (SWD), which are believed to play a vital role in recruiting the adjacent penumbral regions of a reversible injury into the core area of an infarction (Nedergaard and Hansen 1993). SWD result from sustained increases of synaptic glutamate and extracellular potassium ions and typically originate in the ischemic core and spread out to the IP zone. In experimental stroke, SWD lead to a step-wise increase of the ischemic core into the adjacent IP regions within a few hours of an occlusion (Gill et al. 1992). These depolarizations impose a considerable energy demand on the ischemic tissue because re-establishment of ionic gradients requires ATP (Hartings et al. 2003). Consequently, SWD worsen the energy state of the ischemic tissue and contribute to the expansion of the infarct core throughout the period of infarct maturation (Hossmann 1994; Dijkhuizen et al.

1999). Eventually, rapid depolarizations after ischemia cause irreversible neuronal death.

2.2.2 Intracerebral Hemorrhage (ICH)

Intracerebral hemorrhages (ICHs) account for approximately 15% of all stroke cases. In a normal functioning brain, neurons are separated from the blood by the blood-brain barrier, formed by glia. When an intracerebral hemorrhage occurs, blood cells spread across the blood-brain barrier into the surrounding tissue and disturb not only the CBF but also the delicate chemical balance. This disturbance leads to hematoma formation and enlargement and eventually results in a brain edema and a cascade of events in brain tissues (Xi et al. 2006).

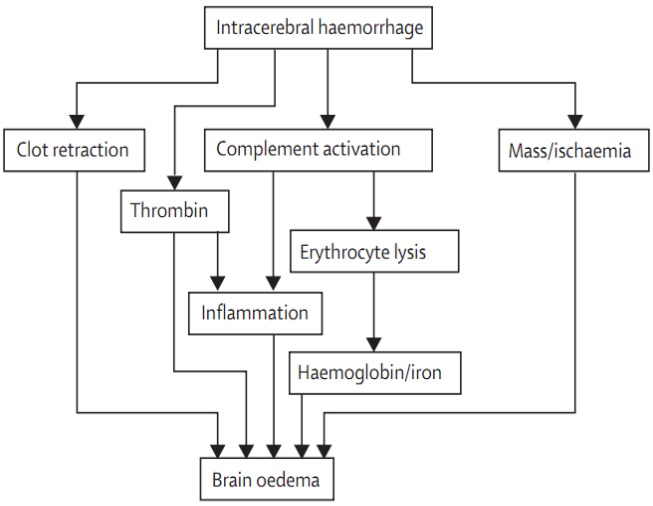


Figure 2.6. Brain edema is formed through several pathways after ICH (from Xi et al. 2006). In the first few hours, hydrostatic pressure and clot retractions form, which is followed by the coagulation cascade and thrombin production. Finally, terythrocyte lysis and hemoglobin toxicity cause brain edema.

Intracerebral hemorrhages result from the rupture of penetrating arteries damaged by hypertension, which is the major cause of spontaneous ICH. Hematomas form

around the bleeding area and do not stop expanding until equilibrium between the bleeding pressure and the increasing intracranial pressure is reached (Ferro 2006). Clinically, 30%-40% of patients suffer from hematoma enlargement within the first 24 hours (Labovitz and Sacco 2001). The growth of hematomas can be attributed to several factors: continuous bleeding and re-bleeding from the burst vessels, bleeding from surrounding compressed vessels, and local clotting defects (Wagner et al. 1996). One major result of this hematoma enlargement is a midline shift and acceleration of neurological degeneration with unknown mechanisms (Zazulia et al. 1999).

Secondary brain damage occurs when an edema forms and involves several phases (Fig 2.6). Early edema develops in the first few hours after a hemorrhage when serum moves from the hematoma into the surrounding tissue due to hydrostatic pressure and clot retraction (Wagner et al. 1996; Xi et al. 2002). After this, a coagulation cascade and thrombin production occurs. Delayed edemas can last for several weeks and are related to erythrocyte lysis and hemoglobin toxicity (Rincon and Mayer 2004). The vasogenic and cytotoxic features of edemas cause the disruption of the blood-brain barrier and lead to programmed cell death, both of which contribute to further brain edema formation (Xi et al. 2006; Yang et al. 1994).

Inflammation aggravates hemorrhagic brain injuries with similar mechanisms as in cerebral ischemia. An inflammatory response in the surrounding brain tissue occurs in the early acute phase and is associated with subsequent hematoma

growth (Xi et al. 2006). Inflammation is also associated with the production of matrix metalloproteinases, which are upregulated in ICHs and cause the a disruption of the blood-brain barrier (Lee et al. 2003).

It is still not clear that whether ICHs cause secondary cerebral ischemia. Studies in rats revealed a decrease of CBF in peri-hematoma areas, but the reduction is temporary and small (Yang et al. 1994). In a dog ICH model, no ischemic area could be defined in the first 5 hours after an ICH (Qureshi et al. 1999). Although animal studies did not indicate a direct relationship between ICH and critical ischemia, it has not been clinically investigated whether ICHs induce local ischemia, especially under high intracranial pressure.

2.3 Outcome Measurements in Animal Stroke Models

Outcome measurements are crucial to the success of translational research and should include both functional responses and pathological evaluations. Generally, neurological function, sensorimotor function, and cognitive status are evaluated as functional outcomes of the animal after stroke; whereas *in vivo* evaluation using imaging techniques and *in vitro* histological evaluation are common pathological measurements.

2.3.1 Functional Outcomes

Neurological deficits are typically difficult to assess directly in rodent models and are thus evaluated by simply checking motor function (Bederson et al. 1986).

More refined methods that assess sensorimotor functions include limb placing tasks, beam walking tests, cylinder tests, and reaching tests (Hunter et al. 2000). Cognitive status is evaluated primarily using the Morris water maze in rat stroke models (D'Hooge and De Deyn 2001).

2.3.1.1 Bederson Scale and Neurological Scoring Scales

The Bederson scale is a global neurological assessment that was developed to measure neurological impairments following stroke (Bederson et al. 1986). The grading scale of the tests ranges from 0 to 3 and examines the forelimb flexion, resistance to lateral push and the circling behavior of the animal. Animals with more neurological deficits, and presumably larger areas of infarctions, will obtain higher scores in the test (Bederson *et al.* 1986). Many iteration scales have been developed and modified since the Bederson scale was developed, including a neurological examination established by Longa et al. that is currently a widely used method in rat ischemic models (Longa et al. 1989; Li and Fisher 2001). The detailed scoring systems in Berderson's scale and Longa's scale are listed in Table 2.4 and Table 2.5, respectively.

Table 2.4. Bederson's neurological examination scoring scales (Bederson *et al.* 1986)

Scores	**Bederson's scale**
0	No observable deficit
1	Moderate, forelimb flexion impaired
2	Severe, decreased resistance to lateral push without circling
3	Decreased resistance to lateral push with circling

Table 2.5. Longa's neurological examination scoring scales (Longa *et al.* 1989)

Scores	**Longa's scale**
0	No neurologic deficit
1	Failure to fully extend forepaw on the affected side
2	Circling to the affected side
3	Falling to the affected side
4	Did not walk spontaneously and had a depressed level of consciousness

2.3.1.2 De Ryck's Behavioral Tests

The De Ryck's behavioral tests examine the sensorimotor functions in rodent stroke models on a 16-point scale that includes 8 tests with each scored from 0 (maximum deficit) to 2 (no deficit) (De Ryck et al. 1989). Six out of the 8 tests specifically evaluated forepaw's functions, including postural reflex, visual placing in the forward and sideways directions, tactile placing of the dorsal and lateral paw surfaces, and proprioceptive placing. The other 2 tests examined the tactile placing of the lateral paw surfaces, and proprioceptive placing of the hindlimbs (Burnett et al. 2006). The detailed scoring system and measurement protocols are listed in Table 2.6.

Table 2.6. (a) Scoring system for limb placing test (De Ryck *et al.* 1989)

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

Table 2.6. (b) Stimulation and functional measures in the test (De Ryck *et al.* 1989)

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 forelimbs and hindlimbs
Proprioceptive	Pushing the rat's paw against the table edge to stimulate limb muscles and joints

2.3.1.3 Walking Test for Hindlimb Function Measurements

Hindlimb testing in rats can be difficult because rodent hindlimbs are not generally used for complex movements. The beam walking test and the grid walking test, however, have been used as reliable measurements of hindlimb functioning in rat MCAo stroke models (Schallert and Woodlee 2005). The walking tests have been shown to objectively demonstrate motor coordination deficits and rehabilitation effects after stroke (Rogers et al. 1997; Bland et al. 2000).

The beam walking test, implemented by Schallert et al., involves scoring the foot slips while a rat transverses of a 2 cm-wide*130 cm-long wooden beam (Fig 2.7a) (Schallert et al. 2002). The beam is elevated 110 cm above the ground and placed horizontally with the home cage at the target end. The rats are placed at one end of the beam, and their foot slips are counted during the walk to their home cage. The maximum score is 6, which indicates no foot slips during the transverse and the minimum score is 0, which indicates that the rat can not balance on the beam. The detailed scoring system for the beam walking test is shown in table 2.7.

Figure 2.7. The beam walking test (left) and the grid walking test (right). In both tests, rats are required to transverse an elevated bar and their foot slips during the walk are counted. (from The University of Cologne 2010).

Table 2.7. Scoring system of the beam walking test.

Beam walking test score	**Description**
0	The rat can not balance and falls down from the beam
1	The rat is unable to traverse the beam but remains perched on the beam
2	The rat falls down while walking
3	The rat can traverse the beam by dragging foot on the affected side
4	The rat traverses the beam with more than 50% foot slips
5	The rat crosses the beam with less than 50% foot slips
6	The rat crosses the beam with no foot slips

In the grid walking test, the rat is placed on an elevated, leveled grid with openings (Fig 2.7b). The grid is 1 m long with irregularly assigned gaps (0.5–5 cm) between round metal bars (Z'Graggen et al. 1998). The number of forelimb and hindlimb placement errors as the rat traverses the grid is recorded (Rogers et al. 1997). The number of faults is then compared to the total number of steps taken and scored using a foot-fault index (Bland et al. 2000). The number of errors can also be rated in a non-parametric grid walk score: 0–1 errors are rated as 3 points, 2–5 as 2 points, 6–9 as 1 point and 10–20 footfalls as 0 points. The grid walk test is a test of motor coordination. The foot fault test has been suggested as a sensitive indicator of detecting impairments in sensorimotor function after ischemia in rodents (Stroemer et al. 1995).

2.3.1.4 Cylinder Test

The cylinder test evaluates neurological impairments by measuring the spontaneous forelimb use in rodent stroke models (Schaar et al. 2010; Schallert et al. 2000). In this test, the rat is placed in a transparent cylinder and observed (Fig 2.8). Rats in the cylinder will rear up on their hindlimbs and explore the surface

with their forelimbs (Hua et al. 2002). The number of forelimb placements with right hand, left hand, and both hands are recorded during their vertical exploration. In most cases, rats with unilateral brain damage will display an asymmetry in forelimb use (Hua et al. 2002).

Figure 2.8. A rat's spontaneous forelimb use is assessed using the cylinder test. Rats with neuron impairments are more likely to use the forelimb on the unaffected site. (from Schaar et al. 2010).

2.3.1.5 Reaching Task

The forelimb/paw reaching task is another qualitative measure in the investigation of skilled forelimb use and motor dysfunctions after brain damage in rodents (Ward et al. 1997; Whishaw et al. 2004). The chamber for this task consists of an elevated center platform with a food pallet on it (Fig 2.9). After food-restriction for two days, a rat is placed into the chamber. The number of attempts, successes, failures and drops during pellet retrieval is recorded for both the ipsilateral and contralateral forelimbs as an indication of impairments (DeVries et al. 2001).

Figure 2.9. In the reaching task, a rat is performing the single pellet retrieval task in a Plexiglas chamber. The rat reaches for banana pellets located contralateral to the trained reaching limb. (from Schaar et al. 2010).

2.3.1.6 Morris Water Maze

The Morris water maze is a widely used cognitive and behavioral assessment that examines the spatial learning and memory system of rats with brain damage. The apparatus for this test consists of a round pool filled with opaque water with temperature maintained at room temperature (Morris 1984). The rat is trained to swim to a hidden platform that is located just beneath the surface of the water. The heading angle during swimming, the time to reach to the platform, and the length of the path travelled are recorded (Fig 2.10). Because of the invisibility of the platform, the animals must depend on their spatial memory and use visual cues outside of the maze to locate the platform, and this aspect of the test assesses the rat's cognitive status (DeVries et al. 2010).

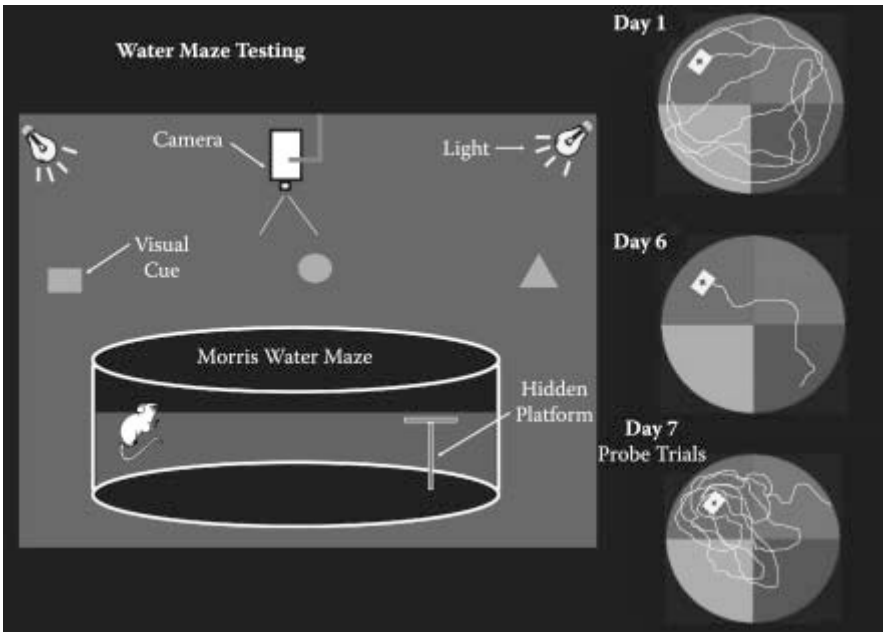


Figure 2.10. Diagrammatic illustration of the Morris water maze system. The rat learns and memorizes from visual cues outside of the maze such as posters, cabinets, and doors to locate the submerged platform. (from Terry 2001).

2.3.1.7 Summery of Function Measurement Tests

Definitely, different function measurement tests have their specific focuses. The summary of above function measure tests is listed in Table 2.8.

Table 2.8. Summery of different function measurement tests (Schaar et al. 2010; Terry 2001)

Name of Test	**Type of Evaluated Function**	**Focus of Function Evaluated**	**Description**
Bederson Scale	Neurological function	Neuron impairments	Simple; easy to distinguish from stroke to non-stroke; but not sensitive in differentiating stroke severity levels; can not specify motor deficits
Longa's Scale	Neurological function	Neuron impairments	Simple; easy to distinguish from stroke to non-stroke; but not sensitive enough in differentiating stroke severity levels; can not

			specify motor deficits
De Ryck's Limb Placing Tests	Sensorimotor function	Sensory & motor impairments of both forelimb & hindlimb	Relatively simple; sensitive in detecting sensory & motor deficits of both forelimb & hindlimb
Beam Walking Test	Motor function	Motor impairments of hindlimb	Simple; sensitive in detecting motor deficits of hindlimb; can not specify motor deficits of forelimb
Grid Walking Test	Motor function	Motor coordination of both forelimb & hindlimb	Relatively complex; not sensitive enough in detecting motor deficits
Cylinder Test	Neurological function	Neuron impairments	Simple; easy to distinguish from stroke to non-stroke; but not sensitive in differentiating stroke severity levels; can not specify motor deficits
Reaching Task	Motor function	Motor impairments of forelimb	Relatively simple; sensitive in detecting motor deficits of forelimb; food restriction required so that may not be suitable in post-stroke research
Morris Water Maze	Cognitive function	Spatial learning & memory	Complex; only spatial learning & memory are tested; can not specify motor deficits

2.3.2 In vivo Examinations Using Imaging Techniques

In vivo examinations of both the underlying mechanisms during the stroke process and the effectiveness of stroke therapies using imaging techniques has been a promising area in recent decades. Imaging techniques play a particularly important role in the differentiation of ischemic strokes and hemorrhagic strokes and provide early indications for prognosis (Ahmed and Masaryk 2004). Traditional methods include computed tomography (CT) and magnetic resonance

(MRI). Recent techniques use ultrasonography to investigate the CBF changes during the stroke process. These imaging techniques may provide crucial clinical implications for therapeutic windows.

2.3.2.1 Rodent Brain Imaging with CT and MRI

CT and MRI are well-established techniques that are used in clinical imaging investigations. Small animal models, however, require a finer spatial resolution than human work. A single photon emission computed tomography (SPECT) was developed for dedicated small animal imaging (Alexandrov et al. 1996). SPECT uses a highly magnified pinhole collimator that can provide millimeter to submillimeter spatial resolution (Meikle et al. 2005). In a study demonstrating SPECT imaging in the ischemic rat brain (Fig 2.11), pinhole SPECT acquisition was performed with a 54×54 matrix, a 1 mm pinhole diameter, and 64 projection views for 30 seconds per projection for a 360° rotation (Seo et al. 2007). Although CT is though to be one of the most accurate methods of distinguishing cerebral ischemia and hemorrhage, it is not sensitive enough to detect ischemic infarctions in the early stages (0.5-12 hours) (Mayer et al. 2000)

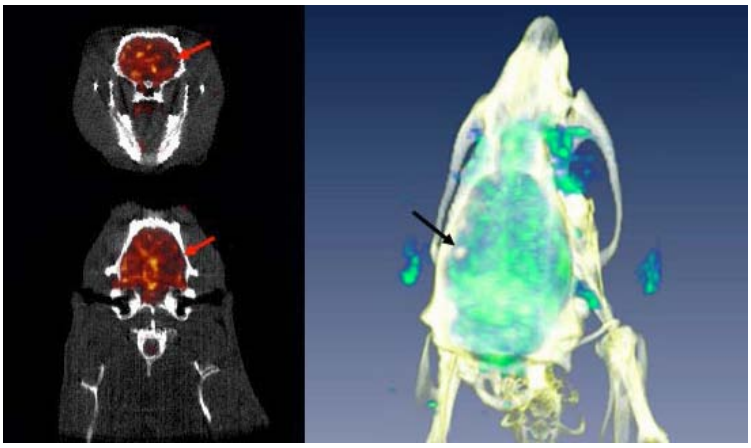


Figure 2.11. Demonstration of the SPECT imaging capability to identify the region of infarction induced by an ischemic stroke in a rat brain. Transaxially, coronally, and three-dimensionally rendered views of a rat brain with infracted regions (arrows) are shown. (from Seo et al. 2007).

High-field MRI is another imaging method that can monitor the *in vivo* changes after brain damage in small animals (Fig 2.12). Developed from traditional MRI, diffusion-weighted (DWI) and perfusion-weighted (PWI) imaging sequences are well established in animal models of cerebral ischemia (Hossmann and Hoehn-Berlage 1995; Jiang et al. 1997). DWI allows real-time measurement and can therefore be used to evaluate the efficacy of stroke therapy (Minematsu et al. 1993). PWI is able to monitor the *in vivo* changes of CBF and demonstrate the benefits of thrombolytic therapy (Jiang et al. 2000). These methods, however, require either contrasting agent infusions or contrast-enhanced magnetic resonance angiography to obtain neurovascular information due to the insufficient temporal and spatial resolution in rodents (Glover and Herfkens 1998; Besselmann et al. 2001). The long acquisition times of angiography infusions can be a limitation for repetitive examinations (Mellin et al. 1994).

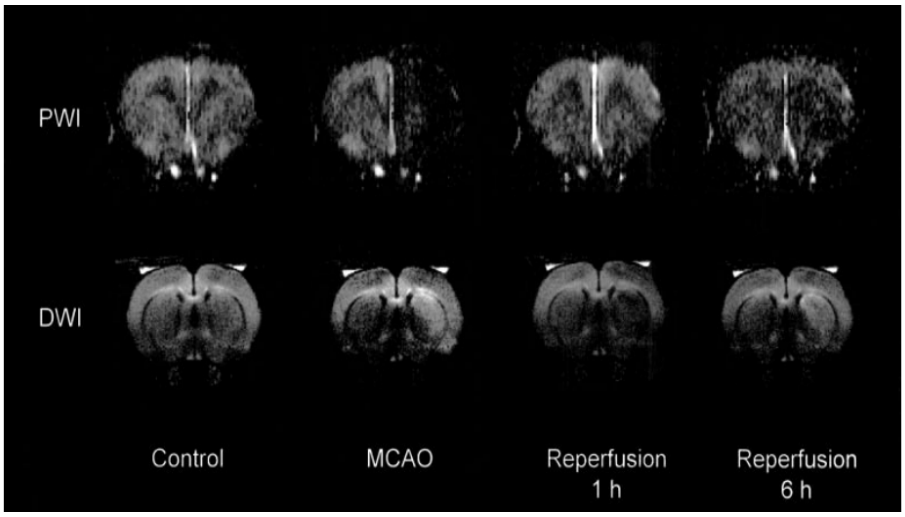


Figure 2.12. MRI images during control, MCA occlusion, 1 hour after reperfusion, and 6 hours after reperfusion. PWI shows the loss and reappearance of imaging signals during the occlusion and reperfusion periods, respectively (upper low), while DWI shows the ion and water homeostasis changes throughout the occlusion-reperfusion process. (from Besselmann et al. 2001)

2.3.2.2 Ultrasonography Techniques

The transcranial Doppler (TCD) ultrasonographic assessment was first introduced by Aaslid et al. and allows adequate penetration through the intact skull (Aaslid et al. 1982). This technique precisely monitors the blood flow velocities of the intracranial blood vessels and has thus become routine in the clinical management of the cerebral blood flow of patients with cerebrovascular diseases (Rigamonti et al. 2008). In animal models, ultrasonography on ischemic brains has been reported in rabbits and rat pups (Els et al. 1999; Bonnin et al. 2008). In these studies, ultrasonography was shown to be able to monitor the real-time velocity changes in large cerebral arteries (Fig 2.13). With this technique, the characterization of the dramatic cerebral hemodynamic changes occurring during cerebral ischemia and reperfusion can be recognized, which may provide important implications for clinical therapeutic strategies.

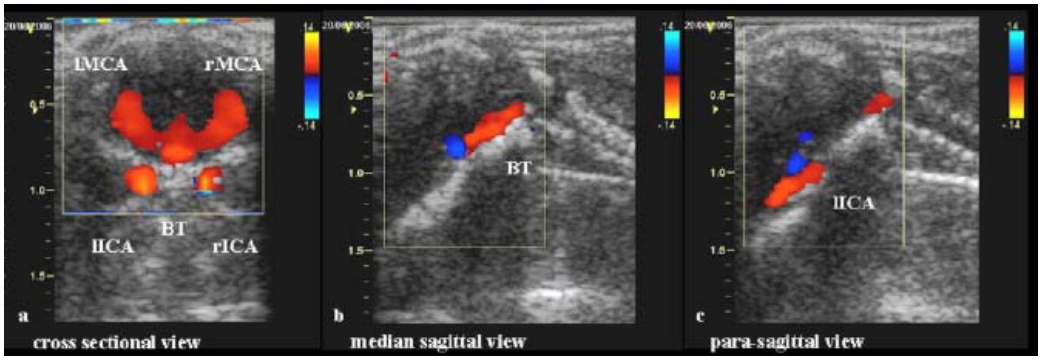


Figure 2.13. Detection of cerebral blood flow in large cerebral arteries using ultrasonography in rat pups. 2-D ultrasound imaging obtained with a cross-sectional image of the rat pup head. The bilateral middle cerebral arteries (MCAs), bilateral internal carotid arteries (ICAs), and basilar trunk (BT) can be recognized (red). (from Bonnin et al. 2008)

2.3.2.3 Summery of Different Imaging Techniques

The advantages and disadvantages of different imaging techniques in stroke research are listed in Table 2.9.

Table 2.9. The advantages and disadvantages of different imaging techniques in stroke research (Iadecola 1999; Bonnin et al. 2008)

Imaging Technique	**Branches**	**Advantages**	**Disadvantages**
CT	SPECT	High resolution; simple; can provide finite image	Not sensitive enough to detect ischemic infarctions in the early stages; invasive radiation; non real-time; less soft tissue contrast
MRI	DWI	Allows real-time measurement	Expensive; contrasting agent required; long acquisition time; can not provide finite vascular information
	PWI	Can monitor the *in vivo* changes of cerebral blood flow	Expensive; contrast-enhanced magnetic resonance angiography required; long acquisition time; non real-time
Ultrasound	TCD	Non-expensive; can monitor the cerebral blood flow velocity in finite vessels; real-time	Can not quantify the brain damage

2.3.3 In vitro Histological Evaluations

Infarct volume has been regarded as the chief metric in focal ischemia and is measured conventionally by quantitative histology. Among a number of histopathological methods, 2,3,5-triphenyltetrazolium chloride (TTC) and hematoxylin-eosin (H&E) stainings are the two most commonly employed (Sicard and Fisher 2009).

2.3.3.1 TTC Staining

TTC is a colorless chemical that it reacts with dehydrogenases, mitochondrial enzymes, in viable cells and results in a "brick-red" color while infarction regions remain white (Fig 2.14). Slice-by-slice lesion areas are summed up to determine the total volume of the infarction. The TTC technique was reported to be reliable 6 to 72 hours after the onset of ischemia (Durukan and Tatlisumak 2009). Prior to 6 hours, the damaged mitochondria may not be sufficient to create a contrast between normal and injured tissue; after 72 hours, a proliferative inflammatory response may obscure the line of demarcation in the periphery of the infarct area and cause an underestimation of the infarct volume (Li and Fisher 2001; Sicard and Fisher 2009). Nevertheless, TTC staining is still preferred over H&E staining because it is much more rapid, easy, and cheaper for infarct volume evaluations in experimental ischemic strokes (Bederson et al. 1986).

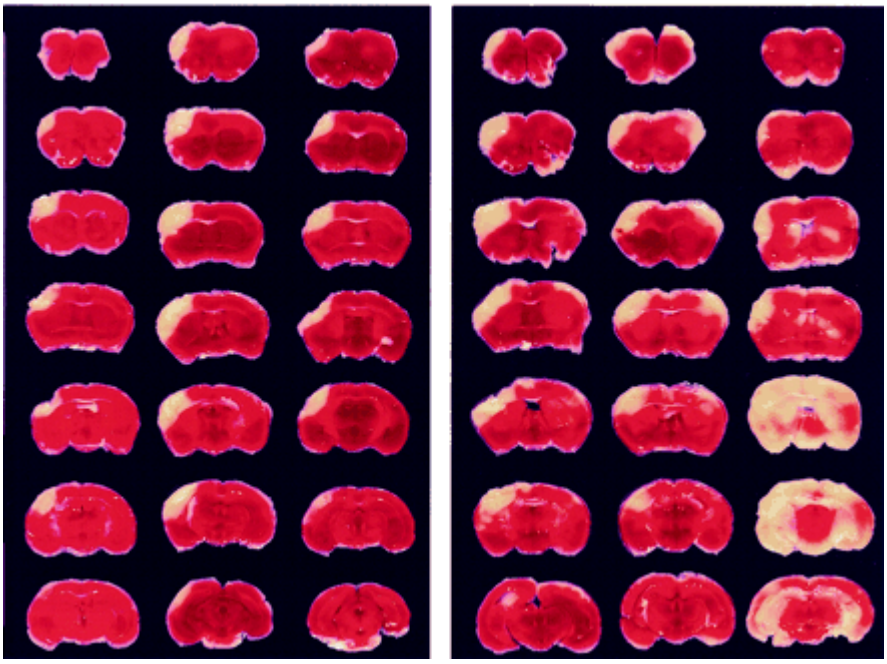


Figure 2.14. TTC staining of rat brain slices with transient (left) and permanent (right) occlusion of left MCA. TTC reacts with dehydrogenases in viable cells and results in a "brick-red" color; the white area indicates the infarction. (from Wang et al. 2001)

2.3.3.2 H&E Staining

H&E staining is a traditional technique that allows both acute and delayed damage in ischemic progress (Garcia et al. 1993). It indicates the acute damage by recognizing cellular swelling and neuropil spongiosis, and identifies the delayed damage by exhibiting eosinophilic (red) features in the neurons that are irreversibly damaged (Garcia et al. 1993; Garcia et al. 1995). Necrotic cell death revealed by H&E staining is characterized by nuclear pyknosis, karyorrhexis, or karyolysis which contain scattered chromatin clumps that are associated with accumulated cytoplasmic eosinophilia and nuclei lacking cellular structures (ghost neurons) (Li et al. 1998). Apoptotic cell death, on the other hand, is characterized by a protuberant cell surface with plasmalemma sealing, which produced membrane-bound apoptotic bodies of roughly spherical or ovoid shape

(Wijsman et al. 1993). 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 H&E-stained brain sections are illustrated in figure 2.15.

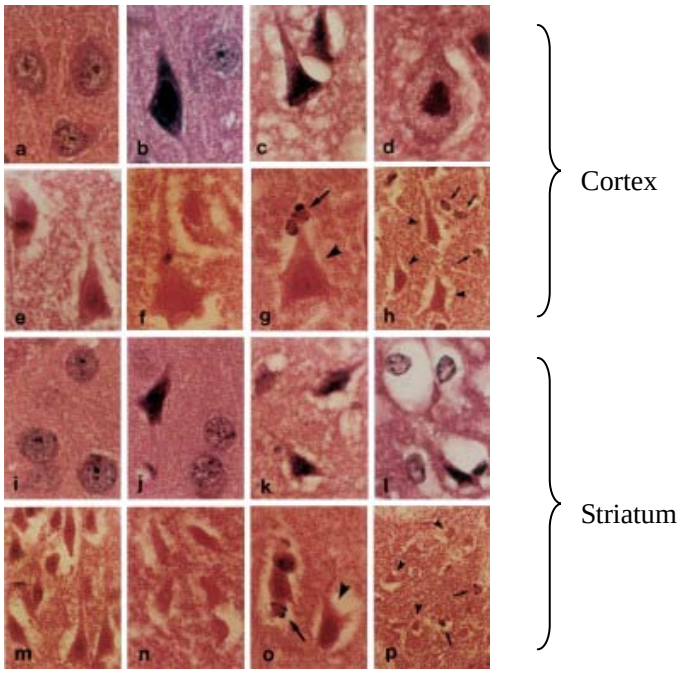


Figure 2.15. H&E staining shows the structural characteristics of intact, injured, necrotic and apoptotic neurons in rats at 46 h after 2 h of MCAo. The affected areas included the cortex and striatum. a–b, i–j: contralateral hemisphere; c–h, k–p: ischemic core. a,i: intact neurons; b,j: non-scalloped shrunken dark neurons; c,k: scalloped shrunken darkneurons; d,l: swollen neurons; e,m: red neurons; f,n: ghost neurons; g–h, o–p: necrotic (arrow heads) and apoptotic (arrows) cells. (from Li et al. 1998)

2.4 Stroke Rehabilitation Training in Animal Models

Stroke survivors typically suffer from persistent disability when the spontaneous recovery after the initial cerebrovascular episode is not sufficient to restore motor function (Stein 2004). Exercise training strategies and therapies have been developed to enhance motor function and reduce handicapping effects. Studies of

the rehabilitation effects of exercise in animal models are directed at providing a scientific rationale for optimizing current stroke rehabilitation practices. Animal studies have found that physical exercise has the potential to augment neuroplasticity processes, which has received relatively little clinical attention (Singh 2004). This improvement appears to depend to a large extent on the exercise-induced elevation of brain derived neurotrophic factor (BDNF), a leading factor in learning and memory, after a brain ischemia (Vaynman et al. 2004). Many researchers suggest that differences in plasticity mechanisms between individuals due to BDNF gene polymorphisms could result in altered levels of plasticity after a stroke (Cheeran et al. 2008; Murphy and Corbett 2009). The following sections introduce common rehabilitation schemes in animal stroke studies and their roles in the regulation of brain neuroplasticity changes.

2.4.1 Treadmill Running

Treadmill running has been used widely as a rehabilitation scheme in both clinical studies and animal models. Many studies on rats have substantiated the beneficial effects of treadmill exercise when performed prior to stroke, compared to the non-exercise group (Wang et al. 2001; Zhang et al. 2010). It was shown that pre-training of at least for two weeks may significantly reduce the infarction volume and edema caused by MCAo (Wang et al. 2001). Similar results were found in another study when exercise was conducted 4, 8, and 12 weeks before the ischemia (Ang et al. 2003). The neuroprotection effect of pre-training may be

explained by an inhibition of phospho-event-related kinase 1/2 over-activation and a reduction in excessive glutamate release during the training (Zhang et al. 2010). Facilitatory effects on motor recovery, compared with spontaneous recovery, were also shown in these studies (Wang et al. 2001; Zhang et al. 2010; Ang et al. 2003).

Studies of treadmill exercise after stroke have also demonstrated the benefits of this rehabilitation. In one study, rats underwent 1 week of treadmill training at 5 days per week and 30 minutes per day, and the results indicate that early training group (24 hours after ischemia) had smaller brain infarction volumes compared with a late training group (1-week after ischemia) and a non-exercise group (Yang et al. 2003). Similar results were shown in studies of both hemorrhagic stroke rats and ischemic stroke rats, where reduced infarct size, improved motor function, and enhanced neuroplasticity were found (Park et al. 2010; Marin et al. 2003; Lee et al. 2003; Leung et al. 2006; Matsuda et al. 2011). Figure 2.16 shows a comparison of infarctions in ischemic rats with or without treadmill exercise. It was noted that exercise suppressed the apoptosis in the dentate gyrus in hippocampus, and enhanced the expression of nerve growth factor in the same region (Lee et al. 2003; Matsuda et al. 2011).

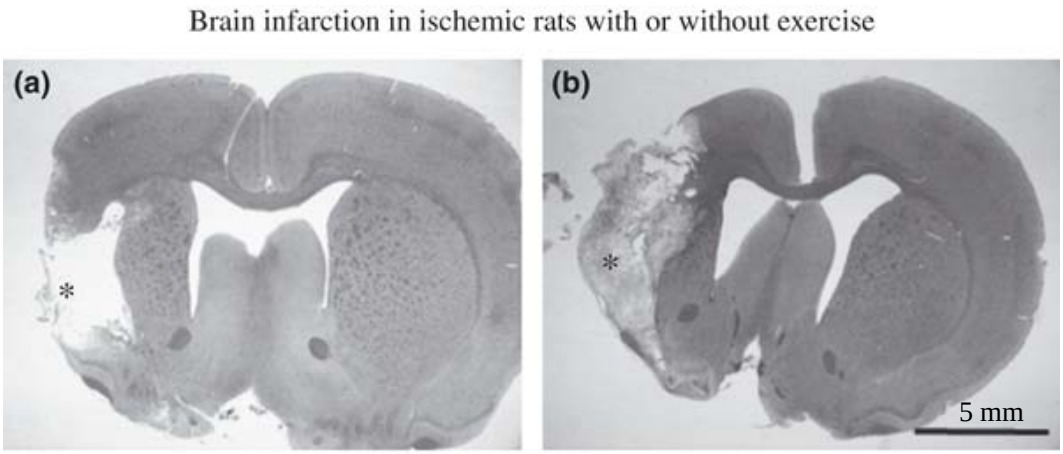


Figure 2.16. H&E staining shows a comparison of brain infarction in ischemic stroke rats with (a) or without (b) treadmill exercise 28 days after training. The brain infarcts were primarily observed in the cerebral cortex including the dorsolateral and lateral cortices (*), and the lateral striatum. (from Matsuda et al. 2011)

Recent studies, however, suggested that the beneficial effects of treadmill training existed only in low-intensity treadmill (15m/min) running rats and that moderate-intensity treadmill (25m/min) would elevate serum corticosterone levels (Soya et al. 2007). Corticosterone elevation is a common sign of chronic stress, which typically causes reduced body weight and spleen atrophy, indicating a negative adaptation to stress (Brown et al. 2007). Moreover, corticosterone was shown to reduce BDNF availability in the rat hippocampus (Schaaf et al. 1998). BDNF supports the survival and growth of many neuronal subtypes and acts as a key mediator of synaptic efficacy, neuronal connectivity and use-dependent plasticity (Kapczinski et al. 2008).

2.4.2 Environmental Enrichment (EE)

Hebb (1947) first discovered that rats placed in a stimulating environment improved problem-solving skills when compared to rats raised in standard

laboratory cages (Hebb 1947). These results prompted further research into the concept of environmental enrichment (EE). An EE, although without standard protocols, often involves social housing in large cages that are filled with inanimate objects for the purpose of increasing sensory, physical, and social stimulation (Janssen et al. 2010). Such objects include, but are not limited to, ladders, ropes, tubes, balls, horizontal boards, swing boards, chains, toys, and on occasions, running wheels (Fig 2.17). Participation in activities within the EE is voluntary. It was shown that rats raised in EEs had greater brain weight, greater neuron size, thicker cortical tissue, a greater degree of dendritic branching, higher dendritic spine frequency, larger synaptic contacts, more perforated synapses, and more synapses per neuron (Nudo et al. 2001; Johansson 2000). The beneficial effects of an EE resulted from morphologic changes in the motor cortex during motor learning (Kleim et al. 1996). Short-term morphologic changes include the immediate expression of early genes, alterations in synaptic efficacy, and long-term potentiation (Rioult et al. 2000), while long-term changes involve an increase in dendritic arborization, spine density, axonal sprouting, and the number of synapses per neuron (Bayona et al. 2005).

Figure 2.17. Environmental Enrichment for stroke rehabilitation in rat models. Ladders, ropes, tubes, balls, horizontal boards, swing boards, chains, and toys are often included in EE.

The application of EE in animal stroke models, however, yielded inconsistent results. Studies have found neutral or negative results with EE when assessing neuron mortality or infarct size (Hicks et al. 2008; Puurunen et al. 2001). Moreover, it was shown that EE caused a marked down-regulation in BDNF gene expression in the contralateral cortex and hippocampus of rats with ischemic strokes (Zhao et al. 2000). Although numerous studies have been conducted on the effects of EE, the results are still controversial; this uncertainty may be due to the complexity and variations of EE setup, which remains a problem in EE research nowadays.

2.4.3 Functional Electrical Stimulation

Functional electrical stimulation (FES), the stimulation of the paralyzed muscle by a specific stimulation pattern, has also been involved in stroke rehabilitation program (Fig 2.18). It was believed that FES was able to accelerate peripheral nerve regeneration and facilitate functional reorganization in hemiplegia after a

brain ischemia (Daly et al. 1996; Chae and Yu 1999). In a rat study, it was shown that at with a long-term program of low-frequency stimulation, the average level of atrophy measured in the soleus and tibialis anterior, was decreased, suggesting that FES might provide significant protection from muscle atrophy (Salter et al. 2003). In addition to changing muscle properties, FES was shown to have similar effects as treadmill exercise in that it suppresses taurine release in the hippocampus of the rat: taurine is a neuroinhibitory transmitter in the central nervous system (Leung et al. 2006). In another study, rats receiving electrical stimulation during brain ischemia showed decreased infarct volume and improved behavioral outcomes (Burnett et al. 2006). FES was also shown to up-regulate BDNF expression in stimulated muscles in rat models (Part et al. 2004).

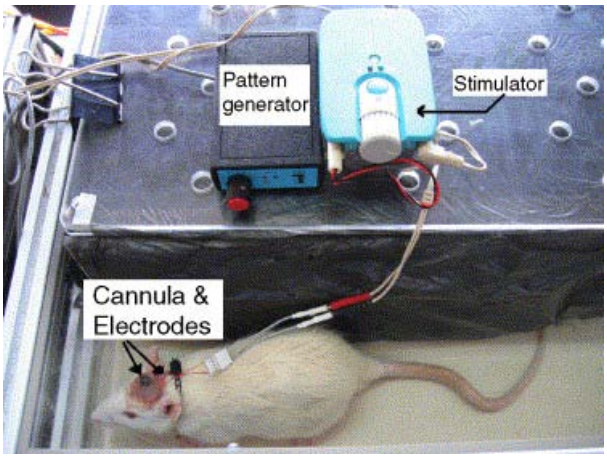


Figure 2.18. Functional electrical stimulation setup. Electrodes were implanted on the affected hindlimb and provided specific simulation patterns after brain ischemia in the rat. (from Leung et al. 2006)

The mechanism of the rehabilitation effects of FES may lie in the active repetitive movement produced by the cyclic electrical stimulation (Chae and Yu 1999). These movements trigger a series of events that facilitate motor relearning and,

consequently, improve voluntary movement: a functional reorganization, where intact areas assume the function of damaged areas; the activation of alternate descending pathways and structures on lesion side; and the activation of pathways and structures in the contralateral undamaged hemisphere (Chae and Yu 1999). It was also suggested that FES could directly activate cutaneous and muscle-tendon afferents and the mechanical events of muscle contractions would have inputs to the spinal cord and, thus, to the brain (Park et al. 2004). Nevertheless, the effects of FES on the impaired central nervous system and damaged brain have not been well studied.

2.4.4 Wheel Running

Developed from EE, wheel running emerges in recent years as a new paradigm of stroke rehabilitation in animal studies. Wheel running is generally regarded to be a type of voluntary exercise in animal models, and it does not activate systemic stress (Yanagita et al. 2007). The wheel setup can be motorized or non-motorized (Fig 2.19), typically with a 1-meter circumference, such that each revolution represent 1 meter in running distance. Although some research suggests that voluntary wheel running is not efficient in reducing brain infarct volume compared to forced treadmill running when performed before stroke (Hayes et al. 2008), Marin et al. concluded that there was no direct relationship between brain infarct volume and motor recovery (Marin et al. 2003). Animal studies have found that voluntary wheel exercise showed superior effects in terms of plastic changes in the dentate

gyrus when compared to forced treadmill exercise (Arida et al. 2004; Collins et al. 2009). Such neuroplasticity was believed to be mediated by BNDF function (Gomez-Pinilla et al. 2002). In accordance with this, Huang and colleagues showed that the up-regulation of BDNF lasted seven and two days in the wheel group and the treadmill group, respectively (Huang et al. 2006). Another study also found that wheel running, when performed before reaching training, facilitated the learning of subsequent challenging reaching tasks after stroke, through the mechanism of up-regulation of BDNF and other modulators of synaptic plasticity (Ploughman et al. 2007). Although most studies have shown positive effects of voluntary wheel exercise, its effectiveness and mechanisms in stroke rehabilitation have not been fully explored at this point.

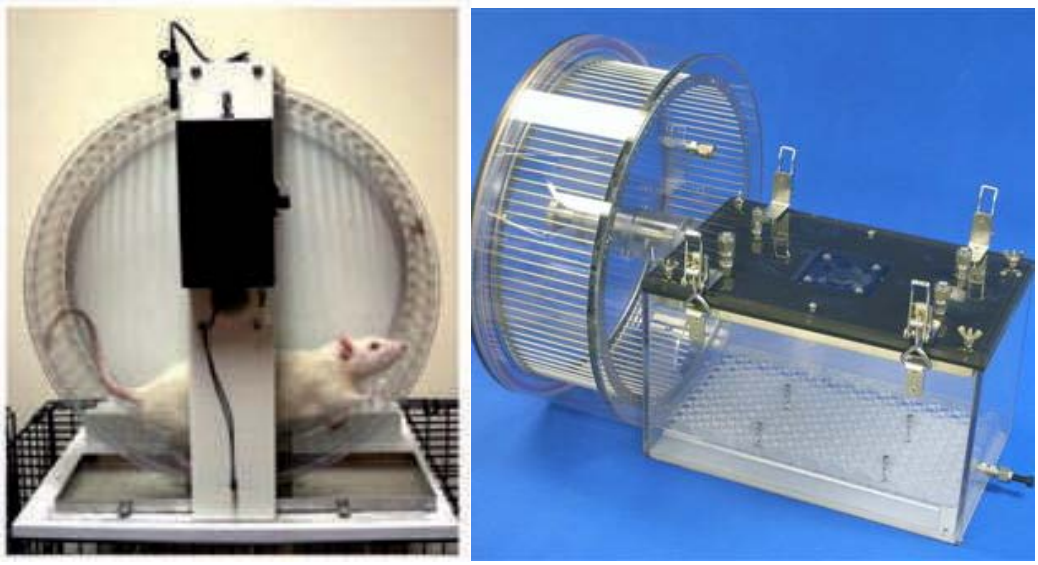


Figure 2.19. Motorized (left) and non-motorized (right) wheel running systems for rats. Usually, a switch connected to the wheel counts and records the number of revolutions. (from Ploughman et al. 2009; Columbus Instruments 2011).

2.5 Neurochemical Changes in Stroke Recovery: the Role of BDNF

BDNF is a powerful differentiation factor that is widely distributed in the central nervous system (Binder and Scharfman 2004). BDNF is essential for neuronal survival and function and, thus, a continuous diminution of BDNF could affect neuronal viability (Skaper 2008). In addition to its effects of supporting neuronal survival, BDNF promotes the formation of dendritic spines and reduces the threshold of long-term potentiation (Schratt et al. 2006; Kiprianova et al. 1999). BDNF also has acute effects on synaptic plasticity and on the facilitation of glutamate, γ-aminobutyric acid, dopamine, and serotonin release (Machado-Vieira et al. 2009). In stroke rehabilitations, BDNF has been identified as playing an important role in various forms of neuroplasticity in both the intact and the damaged brain in the past 10 years (Bekinschtein et al. 2008). It was even suggested that levels of plasticity after stroke could be primarily due to a difference in BDNF concentrations between individuals (Cheeran et al. 2008; Murphy and Corbett 2009).

An acute BDNF administration after an ischemic stroke reduces cell mortality and infarct volume (Zhang and Pardridge 2006). Chronic treatment with BDNF also showed positive effects such as facilitating motor recovery in rats (Schabitz et al. 2007; Muller et al. 2008). In stroke rehabilitation with exercise, many studies have directed a proportional relationship between exercise and BDNF levels (Ploughman et al. 2005; Huang et al. 2006). Elevated BDNF

concentrations have been shown to improve behavioral outcome and cognitive functions in rodent models (Ploughman et al. 2007; Vaynman et al. 2003). BDNF facilitates motor recovery by promoting cortical reorganizion, which is thought to be one of the main effectors of recovery after stroke (Monfils et al. 2005). Exercise increases BDNF levels in the cortex, which may contribute to cortical map reorganization, increased synaptogenesis, enhanced dendritic spine formation and branching, and other forms of neuronal plasticity implicated in recovery after stroke (Monfils et al. 2005; Biernaskie and Corbett 2001).

However, the down-regulation of BDNF appears to have the potential to attenuate recovery processes (Nygren et al. 2006). A clinical study showed that healthy individuals with inhibitors in the BDNF gene exhibited a reduced ability to change cortical maps in response to motor training (Kleim et al. 2006). In animal models, BDNF-mutant mice exhibit impaired long-term potentiation and are unable to learn (Linnarsson et al. 1997). Moreover, the beneficial effects of exercise after stroke are prevented in animals treated with BDNF antisense oligonucleotides (Ploughman et al. 2009).

Together, these findings provide evidence for the role of BDNF in cortical reorganization, motor learning and memory, processes that are likely to be important for functional recovery after stroke. To date, the effectiveness of different exercise regimens on neuroplasticity during stroke recovery is fully understood, and the effects these regimens on BDNF regulation still require for further investigation.

CHAPTER 3

METHODOLOGY

3.1 Structure of the Study

The overall structure of current study can be divided into two parts: (1) a pilot study was conducted to investigate the setup and the cerebral blood flow changes, brain damage areas and neurological deficits in ischemic stroke model and hemorrhagic stroke model; and (2) a rehabilitation intervention study was performed to compare the effectiveness of different exercise interventions in ischemic stroke rehabilitation (Fig 3.1). The ischemic stroke part of ultrasonography study was conduced by Dr. Li Le and the author together, but with different objectives: Dr. Li Le focused on the analysis of cerebral blood flow changes along the ischemia-recovery process (Li L, Ke Z, Tong KY, and Ying M 2010), while the author aimed to investigate the differences of cerebral blood flow changes between ischemic strokes and hemorrhagic strokes so that the hemorrhagic stroke part of ultrasonography study was led by the author and a paper was submitted with respect to this objective.

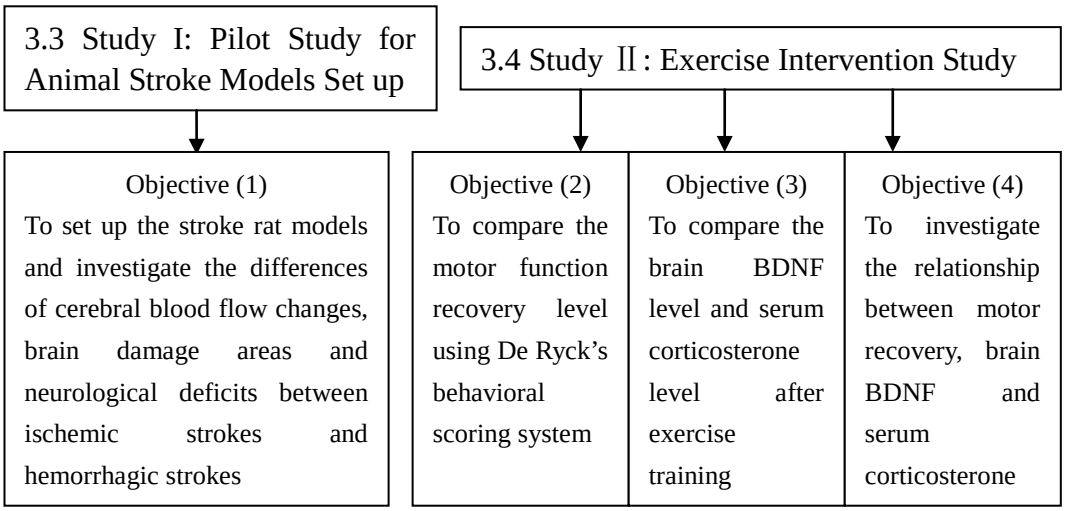


Figure 3.1. Structure and components of the study

3.2 Ethical Consideration

The Animal Ethics Review Committee of the Hong Kong Polytechnic University reviewed and approved the procedures of this study. Animal license from the Department of Health has been issued for this study (Ref no. 08-82) (appendix 1).

3.3 Study I: Pilot Study for Animal Stroke Models Setup

The pilot study was conducted to set up the animal stroke models, and to investigate the cerebral blood flow changes, brain damage areas and neurological deficits in the ischemic stroke model and the hemorrhagic stroke model. Firstly, the duration of cerebral artery occlusion in ischemic stroke model and the duration of exercise intervention were determined. Secondly, the brain-derived neurotrophic factor (BDNF) detection using Enzyme-linked immunosorbent assay (ELISA) technique was validated. Finally, investigations of differences

between ischemic stroke model and hemorrhagic stroke model were conducted.

In the pilot study, 100 young male Sprague-Dawley (SD) rats with body weights 280-320g were used. All rats were provided by the Central Animal Facilities of the Hong Kong Polytechnic Unviersity, and they were housed individually during a 12-hour light/dark cycle with *ad libitum* access to food and water in a temperature-controlled environment (20-22℃). 60 rats were used for the study of occlusion and exercise durations, and the validation of BDNF evaluation technique (section 3.3.1-3.3.4). The remaining 40 rats were used in the investigation of differences between ischemic stroke model and hemorrhagic stroke model (section 3.5).

3.3.1 Accommodation

60 rats would firstly experience a 3-day accommodation upon arrival, during which period they were trained to run on a motor driven treadmill (KN-73, Natsume Ltd., China. Fig 3.2a) as well as on a wheel (manufactured by Kan Kee Sheet Metal Works, Hong Kong. Fig 3.2b). During the accommodation, rats were trained to run on the treadmill at a slope of 0° inclination for three 10-minute sessions, with a 10-minute rest between each session (Nomura et al 2005). The running speed of the treadmill was set at 10m/min, 15m/min, and 20m/min on the first, second, and third day, respectively. A nudge on the back was administered to the rat when it did not move forward on the treadmill (Li et al 2009). For the remainder of each day during the accommodation period, each rat was put in an

individual wheel and let free to run. The circumference of the wheel is 1m, and the wheel is connected to a switch that counts each revolution (Hayes et al 2008). After accommodation, the rats that refused to run on the treadmill or ran less than 600 m/day in the wheel cage would not be selected for the next stage of the experiment. The drop-out rate of accommodation was around 15%. These rats were sacrificed after accommodation and BDNF levels in their hippocampi and lumbers were evaluated as baseline data.

(a) (b)

Figure 3.2. Instrucmentations of exercise intervention. (a) The motor-driven treadmill. The running speed could be adjusted from 0-30m/min. (b) Wheel cage. The rats could either run on the wheel assembly or rest in the other space of the cage.

3.3.2 Effects of Different Durations of Middle Cerebral Artery Occlusion

Focal cerebral ischemia will be induced using the middle cerebral artery occlusion/reperfusion (MCAo/r) model (Longa 1989) in the rats that passed the accommodation. The rats were weighted and anesthetized with chloral hydrate (0.4 mg/kg for induction and 0.02 mg subsequently) by intraperitoneal injection.

The right carotid region was exposed through a midline cervical incision (about 20mm) and the common carotid and external carotid arteries were ligated with a No. 6-0 mm suture. A No. 3-0 monofilament nylon suture with its tip rounded by heating and coated with poly-l-lysine was inserted from the common carotid artery and advanced to a point approximately 17 mm distal to the bifurcation of carotid artery where the proximal segment of the anterior cerebral artery (Fig 3.3) starts, and hence block the blood flow to the MCA. The inserted suture was gently withdrawn 60min/90min/120min after transient occlusion and thus reperfusion was achieved. Core temperature will be maintained by a heating lamp. Enrofloxacin will be injected intramuscularly for prophylaxis after MCAo/r surgery.

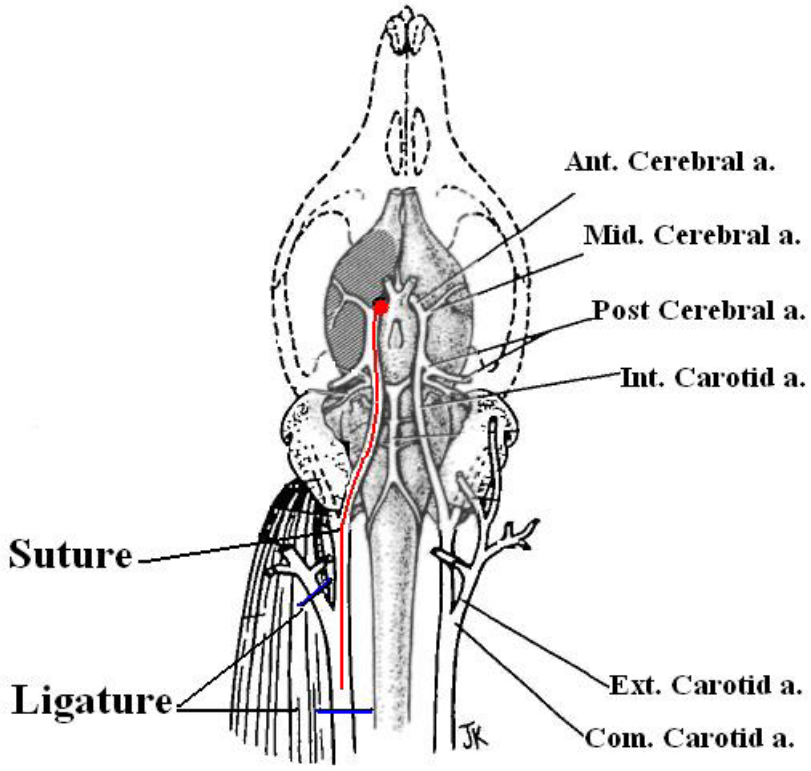


Figure 3.3. The nylon suture (red) was inserted from the common carotid artery and advanced to the proximal segment of the anterior cerebral artery. The common carotid artery and external carotid artery will be ligated (blue) (Longa et al. 1989).

After the MCAo/r surgery, some rats died from severe stroke, while some other rats did not have motor deficits. The mortality and failure rate could be influenced by the occlusion durations (data was shown in Chapter 4). Only survival rats with ischemic strokes were selected for the next stage of experiment. These rats were then randomly divided into two groups: the 7-day group (G1) and the 14-day group (G2). Both group rats were housed individually in wheel cages and let free to run. Daily running distance, body weight and behavioral score were measured and recorded. G1 rats were sacrificed 7 days after MCAo/r surgery, while G2 rats were sacrificed 14 days after the surgery.

3.3.3 De Ryck's Behavioral Test

The evaluation was based on the protocol of De Ryck et al., which is a 16-points scale including 8 tests with each scored from 0 (maximum deficit) to 2 (no deficit), while score 1 means incomplete and/or delayed placing. 6 out of 8 tests specifically evaluates forepaw's functions, including postural reflex, visual placing in the forward and sideways directions, tactile placing of the dorsal and lateral paw surfaces, and proprioceptive placing (Burnett et al 2006), while the other 2 examines the hindlimb's tactile placing of the lateral paw surfaces, and proprioceptive placing. The detail behavioural assessment procedures are described in Table 3.1. Each test was repeated for 3 times and average score was recorded.

Table 3.1 The assessment procedures of De Ryck's behavioral test

Forelimb:

Test 1	Slowly lower the rat toward a table and observe the forepaws streching. At about 10 cm above the table, normal rats would stretch and place both forepaws on the table.
Test 2	With the rat's forepaws touching the table edge, the head of the rat is moved 45^{o} upward while the chin is supported to prevent the nose and the vibrissae from touching the table. Observe whether the rat lost contact with the table. A rat with focal brain lesion may lose contact with the table with the paw contralateral to the injured hemisphere.
Test 3	Forelimb placement of the rat when facing a table edge is observed. A normal rat places both forepaws on the table top.
Test 4	Recorded forelimb and hindlimb placement when the lateral side of the rat's body is moved toward the table edge.
Test 5	Rat is placed on the table and gently pushed from lateral toward the table edge. A normal rat will grip on the edge, but an injured rat may drop the limbs contralateral to the injured hemisphere.
Test 6	Same as Test 5, but the rat is pushed frontally toward the table edge.

Hindlimb:

Repeat Test 4 and Test 5 and observe the hindpaws placing behaviors.

3.3.4 Brain-derived Neurotrophic Factor (BDNF)

The rats were sacrificed by intracardial perfusion with deep anesthesia, and the bilateral hippocampi and lumbar spinal cord L3-L5 were harvested and stored separately in 1 ml ice-cold lysis buffer and homogenized. The homogenate was diluted 1:5 in Dulbecco's phosphate-buffered saline. 1 μl of 1N HCl for each 50 μl of diluted sample was added and the pH was checked using pH paper to ensure that it was less than 3.0. The solution was then mixed and incubated for 15 minutes at room temperature. After that, the diluted sample was neutralized by adding 1 μl of 1N NaOH per 10 μl of solution. The pH was checked to ensure that it was approximately 7.6. The acid-treated sample was centrifuged for 3 min at

14,000 rpm (Cechetti et al 2008). The supernatant was collected and stored at -20 ℃. BDNF protein was assessed by EMax® ELISA kit (Promega, WI, USA) according to manufacturer's recommendations, and the detailed experimental procedure was listed in the Appendix 2. The results of hippocampal and lumbar BDNF levels for the baseline (B, n=9), G1 (n=9), and G2 (n=9) groups rats, were shown in Chapter 4.

3.3.5 Investigation of Ischemic Stroke Model and Hemorrhagic Stroke Model

This study was to investigate the differences between ischemic strokes and hemorrhagic strokes in the aspects including cerebral blood flow changes and sesorimotor deficits and recovery after strokes. 40 rats were used to study the cerebral blood flow changes, the brain damages and the neurological deficits in the ischemic stroke model and the hemorrhagic stroke model. 24 hours after their arrival, all rats underwent an ultrasonography scanning for the evaluation of cerebral blood flow velocity and data were recorded as baseline. After the baseline scanning, rats were randomly distributed into two groups: Ischemic stroke group (n=26), and Hemorrhagic stroke group (n=14), as the successful and survival rate of ischemic stroke surgery with 90-minute occlusion is around 50% of that of hemorrhagic stroke surgery. The ischemic stroke group rats received MCAo/r surgery and ultrasound scanning during the occlusion period, after reperfusion, and daily scanning for 4 concessive days after the surgery; whereas the hemorrhagic stroke group rats received intracerebral hemorrhage (ICH)

surgery and ultrasound scanning immediately after the surgery, as well as daily scanning for 4 concessive days after the surgery. Their motor recovery level was evaluated with De Ryck's behavioral scoring system on a daily basis. The experiment procedure is shown in figure 3.4.

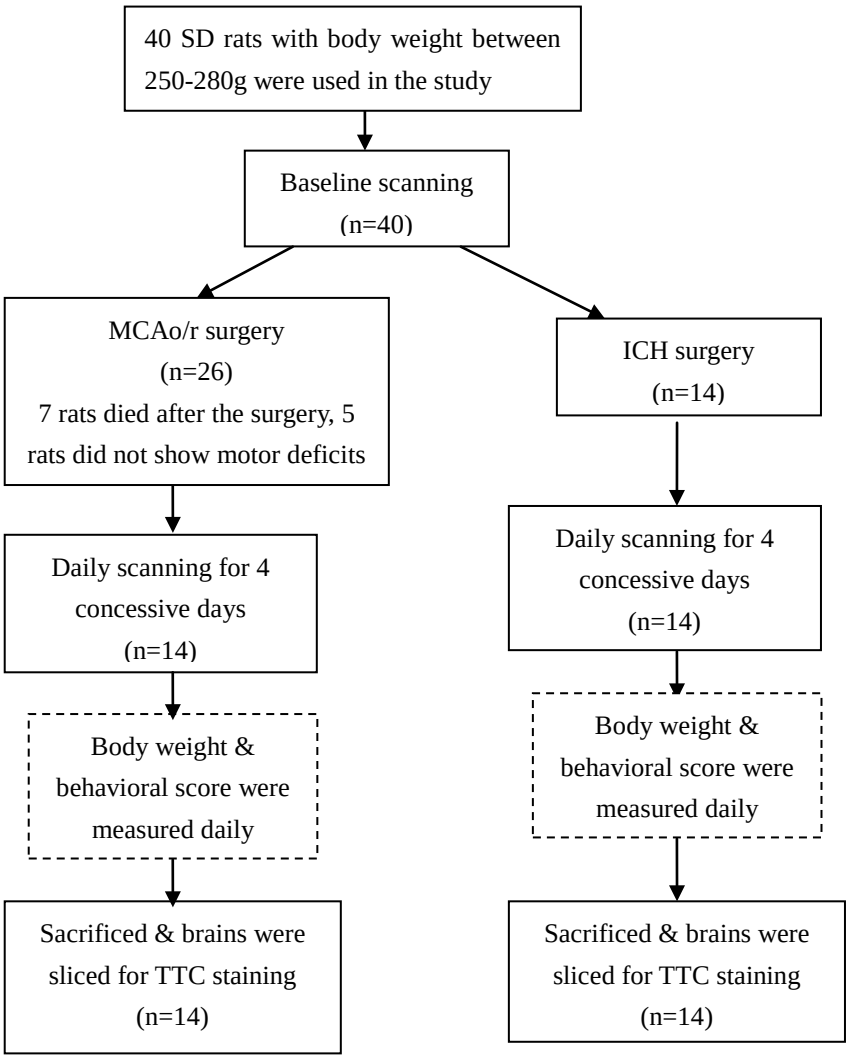
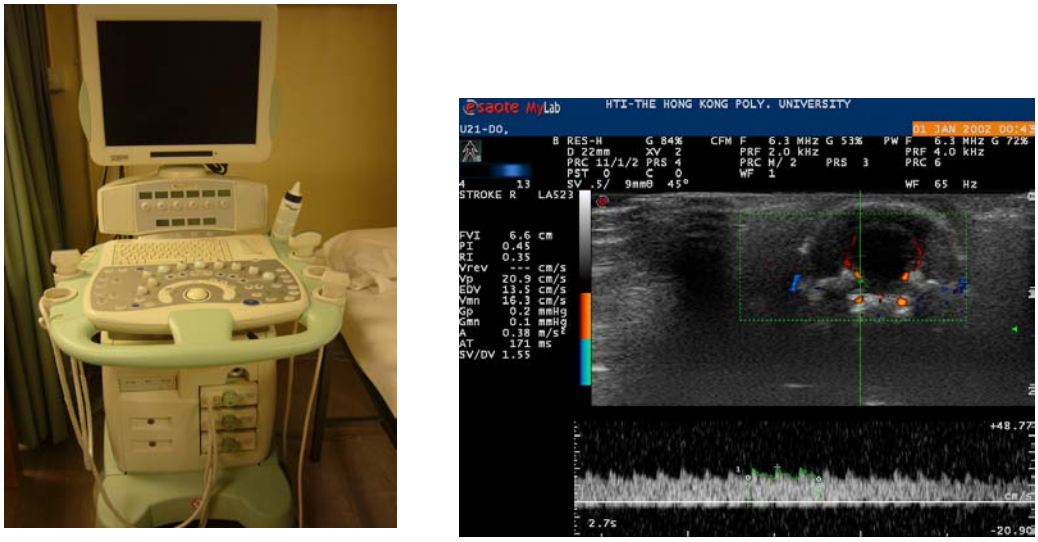


Figure 3.4. CONSORT flowchart of the experiment procedure of ultrasound scanning study.

3.3.5.1 Ultrasonography Scanning Parameters

An Esaote MyLab 70 X-view ultrasound unit in conjunction with a 13 to 4 MHz

linear transducer (Esaote, Genova, Italy) was applied in this study (Fig. 3.5a). The applied ultrasound unit is able to provide clear vessel distribution *in vivo* in small animals such as rats (Fig. 3.5b). Ultrasonic frequency was set at 6.3 MHz, pulsed repetition frequency (PRF) at 4 kHz and the frame rate was 65 frames per second. Imaging depth was set at 22 mm when applying zoom. The peak systolic velocity (Vp), end diastolic velocity (Edv), and the time-averaged maximum blood flow velocity (Vmn) can be directly read and recorded from the ultrasound window (Li L, Ke Z, Tong KY, and Ying M 2010).



(a) (b)

Figure 3.5. The Esaote MyLab 70 X-view ultrasound unit (a) and the 2-D image provided by the ultrasound unit (b). The ultrasound unit provides clear vessel distribution in a rat brain (red: blood flow towards the probe; blue: blood flow away from the probe). The green cursor indicates the vessel measured, and the measured information was shown in the left column, including the peak systolic velocity (Vp), end diastolic velocity (Edv), and the time-averaged maximum blood flow velocity (Vmn).

3.3.5.2 Ultrasonography Reliability Test

As ultrasonography was newly applied in the measurement of cerebral BFV in

small animals (Bonnin et al. 2008), the reliability of the technique was test using an intraclass correlation coefficient (ICC) method. To check whether the technique was operator-dependent, the $ICC_{2,1}$ model was used. Briefly, two separate and independent operators measured the BFV in a randomly selected artery in six rats under same condition at the baseline stage, and the measurement was repeated alternatively for a total of three times with 10 minutes interval between measurements. The results were collated double blind and the test-retest repeatability was evaluated using $ICC_{3,1}$ model by comparing the results from the same operator (Li L, Ke Z, Tong KY, and Ying M 2010). According to Meyers and Blesh (Meyers and Blesh 1962), the reliability can be classified into: high ($\geq$0.9); good (0.80~0.89); fair (0.70~0.79); low ($\leq$0.69).

3.3.5.3 Intracerebral Vessels

Five cerebral arteries were identified and examined in the ischemic stroke study, including the bilateral common carotid arteries (CCAs), the bilateral middle cerebral arteries (MCAs), and the single basilar artery (BA). Another two cerebral arteries, which are the bilateral posterior cerebral arteries (PCAs), were included in the hemorrhagic stroke study as it was found that the cerebral blood flow velocity (BFV) in the PCA were also measurable and interrelated with hemorrhagic strokes. After anaesthetized with 10% chloral hydrate (0.4 mg/kg for induction and 0.02 mg subsequently), the rat was fixed on a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA) with the hair on the head razed.

Conduction gel was used between the probe and the head to improve the conductivity, and the probe moved back and forward on the skull at coronal plane for the detection of the PCAs, the MCAs, and the BA (Fig. 3.6). The bilateral PCAs (Fig. 3.7B) and MCAs (Fig. 3.7C) were identified by their symmetrical features, and the PCAs were towards the neck with a horizontal distance between left and right arteries around 0.72 cm, while the MCAs were towards the nose with distance around 0.85 cm. The BA (Fig. 3.7D) was recognized in the middle of skull base at coronal plane, whereas the bilateral ICAs (Fig. 3.7A) were identified when the probe was placed at sagittal plane.

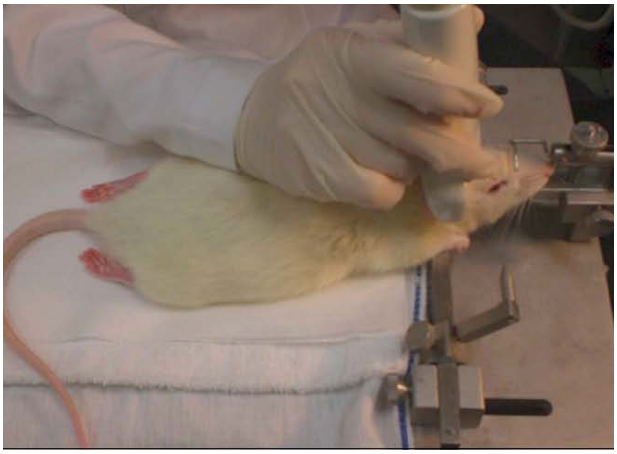


Figure 3.6. The rat was fixed on a stereotactic frame and the probe moved back and forward at coronal plane when detecting the MCAs, the PCAs, and the BA (Li L, Ke Z, Tong KY, and Ying M 2010).

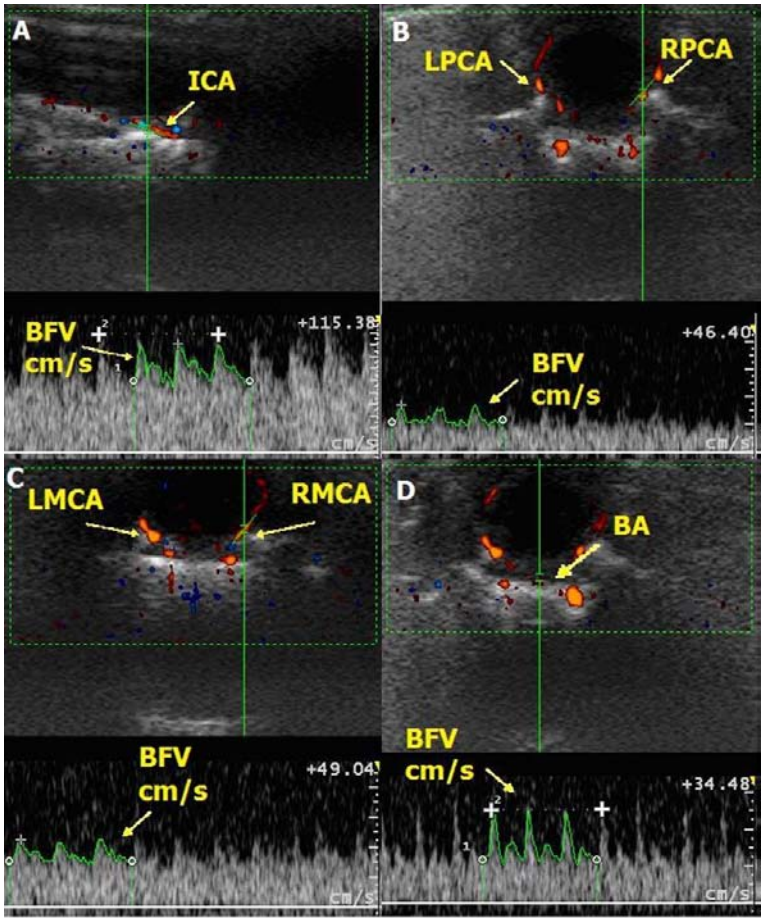


Figure 3.7. Color-coded and 2-D ultrasound imaging of the cerebral arteries in the rat. (A) The internal carotid artery measured at longitudinal direction (ICA, indicated by the arrow) (B) The left and right posterior cerebral artery measured at cross-sectional direction (LPCA and RPCA, indicated by the arrow) (C) The left and right middle cerebral artery measured at cross-sectional direction (LMCA and RMCA, indicated by the arrow) (D) The basilar artery measured at cross-sectional direction (BA, indicated by the arrow).

3.3.5.4 Ischemic Stroke Rats Group

26 rats received 90-minute MCA occlusion-reperfusion surgery in this study, which surgery was described in previous section 3.3.2. During the occlusion, ultrasound scanning was performed to verify the position of the filament (Fig. 3.8), and also measure the BFV in the LMCA, the RMCA, the BA, and the LICA as the RICA was permanent ligated in the surgery and blood flow in the RICA was not allowed. After reperfusion, ultrasound scanning was also conducted

within 30 minutes, and the rat was sent back to its original cage afterwards. 24 hours after the surgery, the survival rats underwent the De Ryck;s behavioral test, which was described in previous section 3.3.3. The rats that either died after the surgery or did not show motor deficits were dropped out. The measurements of body weight and behavioral score were performed on the remaining rats on a daily basis before the ultrasound scanning. The measurements and scanning lasted for 4 concessive days, and rats were sacrificed by intracardial perfusion for brain Triphenyltetrazolium chloride (TTC) staining after the experiment.

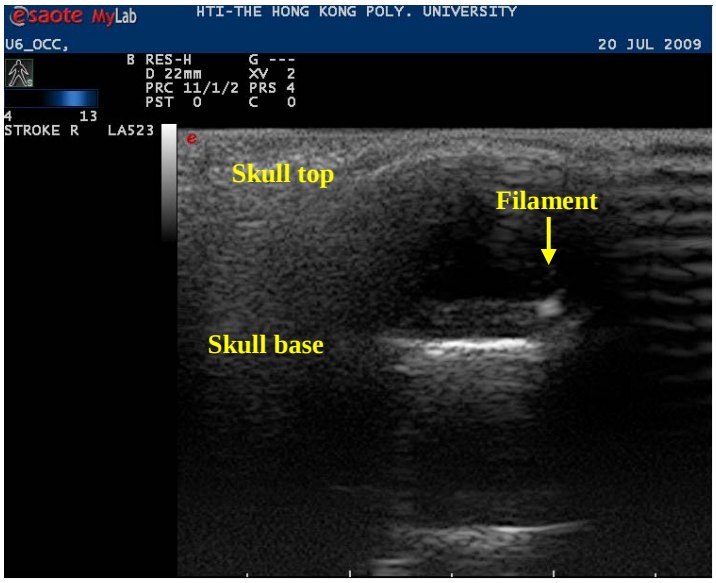


Figure 3.8. The ultrasonography image verified the position of intraluminal filament (arrow pointed).

3.3.5.5 Intracerebral Hemorrhage (ICH) Stroke Rats Group

14 rats were used in this study. After their baseline scanning, all rats received an ICH surgery and induced hemorrhagic stroke after the surgery. The ICH surgery was based on rat hemorrhagic model developed by Rosenberg and coworkers (Rosenberg et al. 1990) which tried to mimic the situations in the human

hemorrhagic strokes (MacLellan et al. 2010). Briefly, the rat was placed and the head was fixed in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA) after anaesthesia with 10% chloral hydrate (0.4 mg/kg for induction and 0.02 mg subsequently). After a midline scalp incision, a burr hole was drilled through the skull (Fig. 3.9C), and a 25-gauge needle attached to a 2-µl Hamilton microsyringe (Fig. 3.9A) was inserted into the right striatum (4.0 mm lateral to midline, 0.2 mm posterior to coronal suture of the bregma (Fig. 3.9B), and 6.0 mm below the surface of the skull). Collagenase (VII-S; Sigma-Aldrich, St. Louis, MO) dissolved in saline was infused through the needle at a dosage of 0.23 units in 1 µl for 10 minutes. After infusion, the needle was slowly withdrawn and the hole was sealed with bone wax, the wound was sutured, and the animal was then transferred to the ultrasound lab for the first scanning after the surgery. Throughout the ICH surgery and ultrasonography measurement a heating lamp was used to keep the body temperature steady to prevent hypothermia.

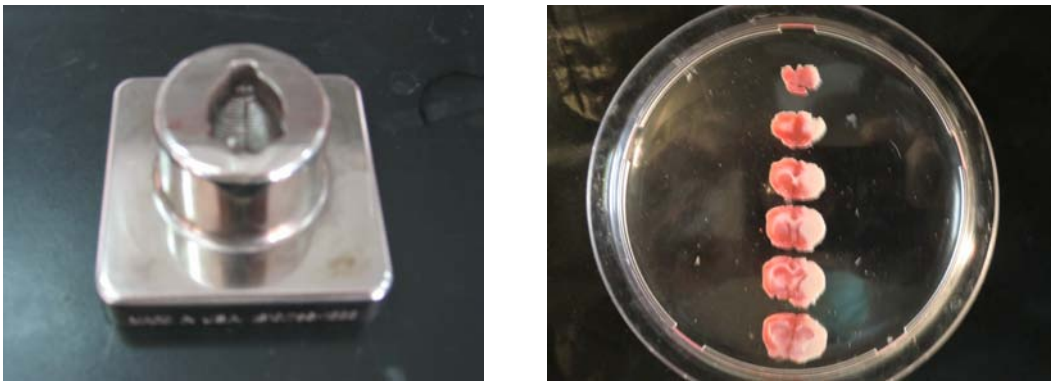
Figure 3.9. The ICH experiment procedure. (A) The rat was fixed in a stereotactic frame under anesthesia; (B) after a midline scalp incision, the bregma was exposed; (C) a burr hole was drilled at 4.0 mm lateral to midline, 0.2 mm posterior to coronal suture of the bregma; (D) a 25-gauge needle was inserted 6 mm below the surface of the skull to inject 0.23 units collegenase into the rat's striatum.

The post-surgery scanning started from 30 minutes after the ICH surgery, and was conducted daily for 4 concessive days. The measurements of body weight and behavioral score were also performed on a daily basis before the ultrasound scanning. All the rats were sacrificed by intracardial perfusion for brain TTC staining after the experiment.

3.3.5.6 Triphenyltetrazolium Chloride (TTC) Staining & Brain Infarct Volume

After sacrifice, the rat's brain was rapidly removed from the cranium and dissected into 2 mm sections using a brain matrix (RBM-4000C; ASI Instruments

Inc., Houston, Texas, USA) as shown in figure 3.10. The brain slices were incubated in fresh 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution (Sigma-Aldrich Co., St. Louis, Missouri, USA) at 37 ºC for 30 minutes. The sections were subsequently immersion fixed using 2% paraformaldehyde solution (Patel et al. 1999). The stained brain slices were photographed with a digital camera and images were analyzed by software (UTHSCSA Image Tool for Windows version 3.00, San Antonio, Texas, USA). Infarction or hematoma areas on each slice were calculated and the total volume of infarction or hematoma was also integrated.



(a) (b)

Figure 3.10. The brain matrix (a) and the brain slices taken from cerebral ischemic rat (b). TTC reacts with dehydrogenases in viable cells and results in a “brick-red” color, and the white area indicates the infarction.

3.3.5.7 Statistical Analysis

Two-way repeated analysis of variance (ANOVA) with Bonferroni Post-hoc test was used to evaluate the BFV changes in the baseline-stroke-recovery procedures in both ischemic stroke group and hemorrhagic stroke group. Multivariate analysis of covariance (MANCOVA) with Bonferroni Post-hoc test was used to

compare the time-averaged maximum (Vmn) BFVs in five cerebral arteries between the ischemic stroke group and the hemorrhagic stroke groups (Tabachnick and Fidell 1996). Results are expressed as mean ± standard deviation (SD) and the level of statistical significance is set at 0.05.

3.4 Study Ⅱ: Comparison of the Effects of Different Exercise Interventions as Stroke Rehabilitation Tools in a Rat Cerebral Ischemia Model

This study was to compare the effectiveness of voluntary exercise, involuntary exercise, and forced exercise as stroke rehabilitation interventions. Specifically, their effects on facilitating motor function recovery and regulating brain BDNF level and serum corticosterone level were investigated. In this study, all the experiments were performed by the author and the results have been published on journal PLoS One (Ke Z, Yip SP, Li L, Zheng XX, Tong KY (2011)). In this study, 150 young male SD rats were used. Rats were provided by the Central Animal Facilities of the Hong Kong Polytechnic University. The rats were weight 280-320g upon arrival and were housed individually during a 12-hour light/dark cycle with *ad libitum* access to food and water in a temperature-controlled environment (20-22℃). The rats were randomly assigned into four groups:

1. Control group (Con) – stroke rats with no exercise intervention;

2. Voluntary exercise group (V-Ex) – stroke rats with voluntary wheel running exercise;

3. Involuntary exercise group (I-Ex) – stroke rats with involuntary muscle contraction exercise by functional electrical stimulation (FES);

4. Forced exercise group (F-Ex) – stroke rats with forced treadmill running exercise.

The detailed experiment procedures were shown in Figure 3.11. Briefly, all the rats were firstly trained to run on the treadmill and in the wheel as accommodation. The accommodation lasted for three days, and rats that failed to run the minimum amount of distance of the treadmill (600 m) and in the wheel (600 m) daily were excluded. Rats that met the minimum distance requirement after the accommodation period (n=117) were randomly divided into two groups: 27 rats in the involuntary exercise group (I-Ex) with electrode implantation and received muscular electrical stimulation after stroke, and 90 rats in the other group without electrode implantation and received other rehabilitation interventions after stroke. After 3 days rest, the I-Ex rats (n=27) and other 90 rats were induced to suffer from ischemic stroke with intraluminal suture middle cerebral artery occlusion/reperfusion (MCAo/r) model. Rats with successfully induced stroke (n=57) were divided into four groups: I-Ex (n=14), voluntary wheel exercise group (V-Ex, n=14), forced treadmill exercise group (F-Ex, n=15), and control group (Con, n=14). The three exercise groups (V-Ex, I-Ex, and F-Ex) received their prescribed rehabilitation intervention for seven consecutive days (I1-I7), while Con rats were put in standard cage and allowed spontaneous recovery after stroke. The sample size estimation was based on previous literatures using animal

models in the stroke rehabilitation studies (Marin et al. 2003; Park et al. 2004).

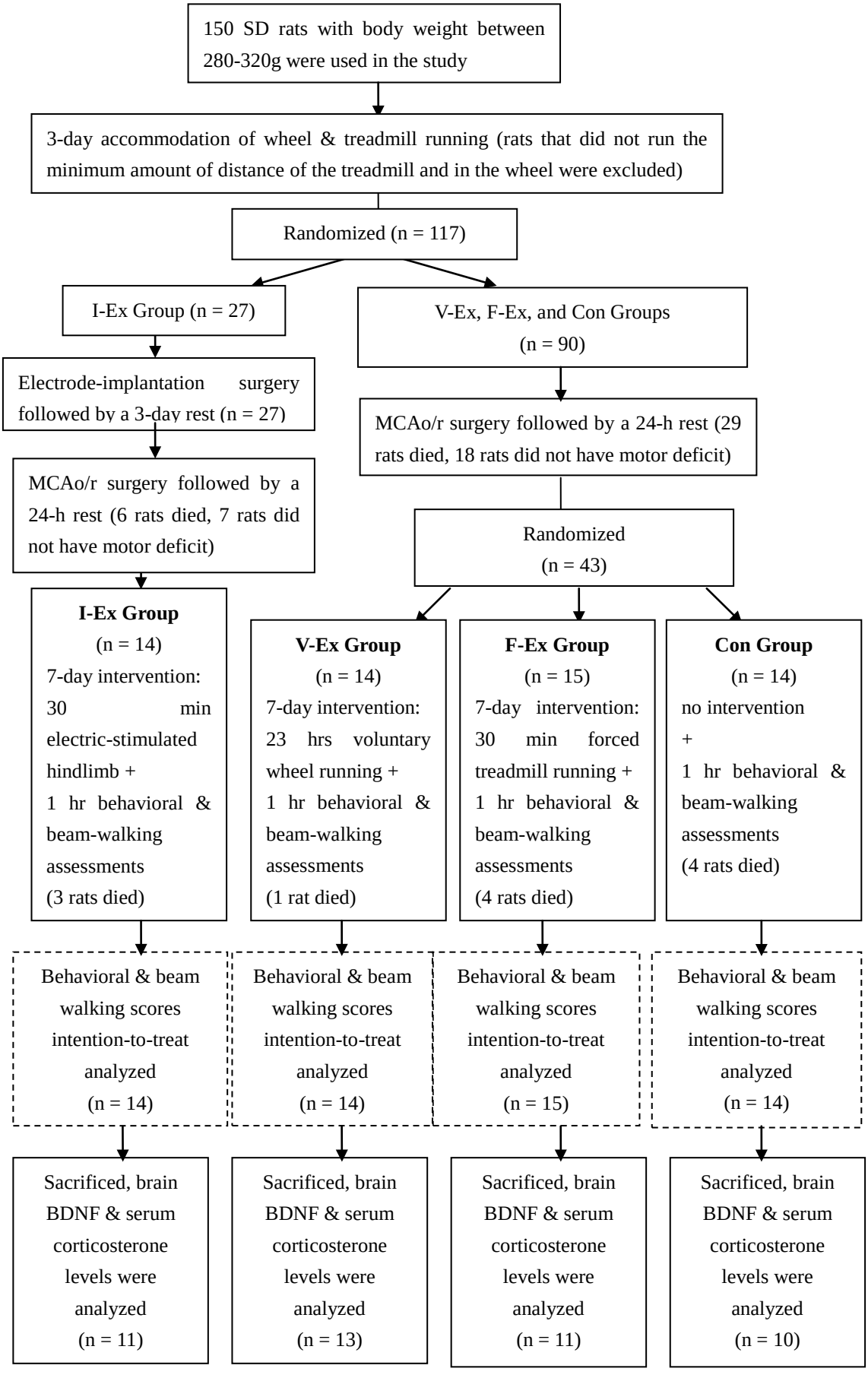


Figure 3.11. CONSORT flowchart of the experiment procedure of study Ⅱ.

3.4.1 Implantation Surgery

Electrode-implantation surgery was carried out on the I-Ex after the rats were anaesthesized with 10% chloral hydrate (0.4 mg/kg for induction and 0.02 mg subsequently). Hair on the left hindlimb and on the head was removed. Incisions were made on the skull to expose the bregma, and on the left hindlimb to expose the muscle pairs namely tibialis anterior (TA) and medial gastrocnemius (MG). Four teflon-coated stainless steel wires (AW633, Cooner Wire, USA) were passed subcutaneously from the skull to the incisions on the limb. One end of each wire was fixed by stripping insulation off and looping them around the bellies and tendons of TA and MG, respectively, and the other end was soldered to a 4-pin connecter and fixed on the skull with four screws, which were implanted on the surface of the skull, and fixed with dental cement (Megapress, Megadental GmbH, Germany) (Marqueste et al 2002; Leung et al 2007). After suture, thresholds of stimulation voltages that allowed TA and MG contraction were recorded and were 1.5 – 6 V (4.3 V ± 1.6 V) and 2 – 8 V (5.6 V ± 2.2 V) respectively (S8800 Stimulator, Astro-Med, USA.) when the rat were still under anesthesia. The painkiller, Buprenorphine (0.1 – 0.3 mg/kg) was prescribed immediately after surgery. The rats were allowed to recover for 3 days before inducing stroke by middle cerebral artery occlusion. Figure 3.12 shows the details of the electrode implantation surgery.

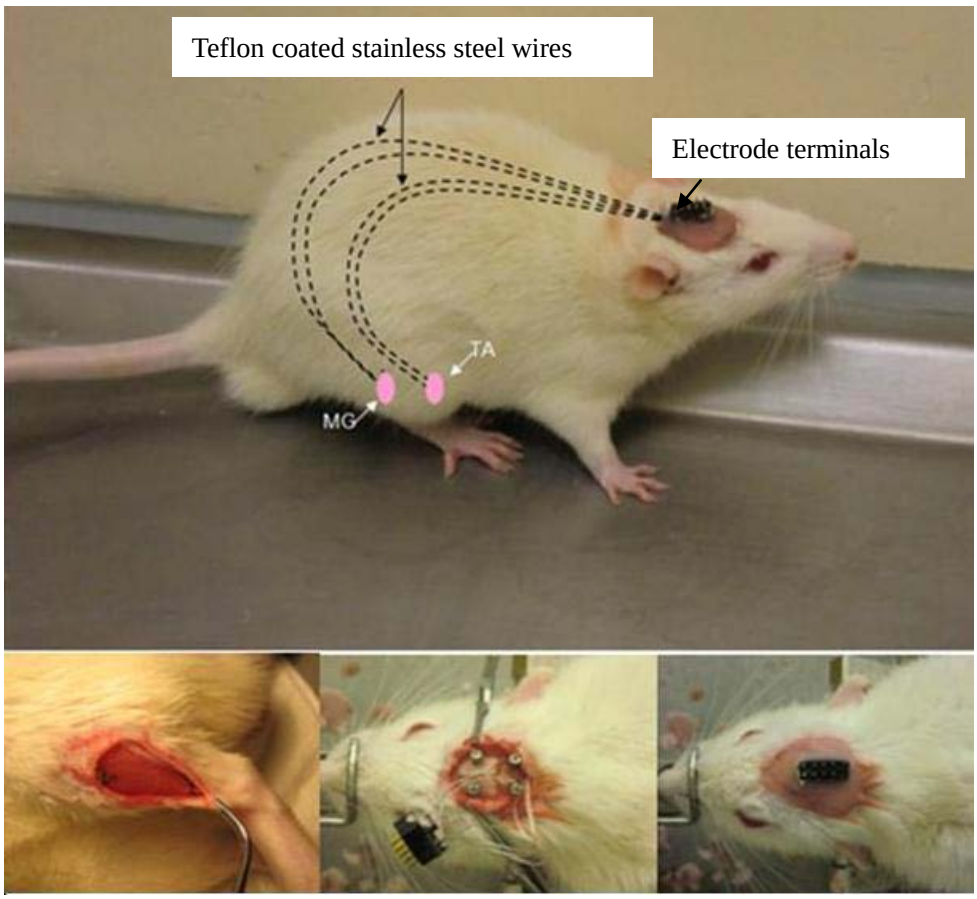


Figure 3.12. The implantation surgery procedure. I-Ex group rats received implantation of electrodes: four teflon-coated stainless steel wires are attached to the muscle bellies of TA and MG on the affected hindlimb, and the screws and headset for fixing the electrodes wire on the skull.

3.4.2 MCAo/r Surgery

All group rats were induced ischemic stroke using the focal cerebral ischemia model which had been validated in the pilot study. The rat's right MCA was blocked by a 3-0 monofilament nylon suture with rounded tip for 90 minutes, and allowed reperfusion by withdrawing the suture. The damages produced in the brain included striatum and frontoparietal cortex which was shown in figure 3.13.

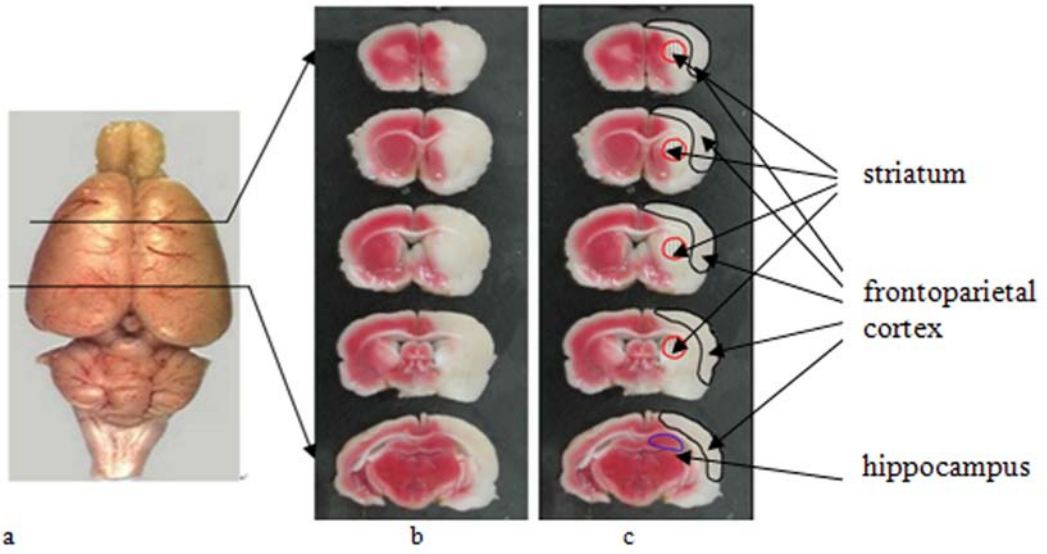


Figure 3.13. The brain infarction revealed by triphenyltetrazolium chloride (TTC) staining 7 days after MCAo/r surgery. (a) Rat brain (b) original TTC image of brain infarction 7 days after the 90 minutes middle cerebral artery occlusion, and each slice was 2 mm in thickness; and (c) image with labels. TTC reacts with dehydrogenases in viable cells and results in a "brick-red" color, and the white area indicates the infarction. It was clearly detected that ipsilateral motor cortex and striatum were affected with middle cerebral artery occlusion.

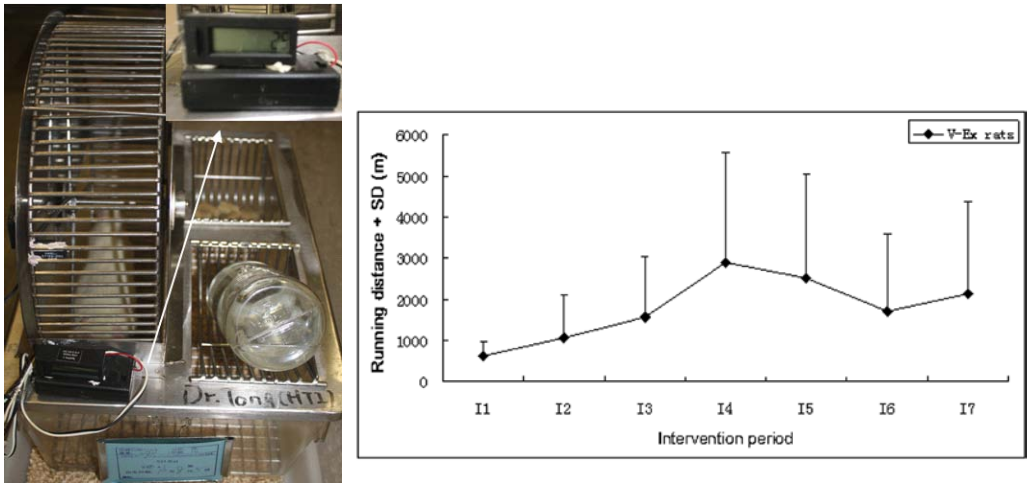
3.4.3 Rehabilitation Interventions

Twenty-four hours after the MCAo/r surgery, the exercise groups started their prescribed exercise interventions, while the control group rats received no intervention and stayed in the standard cage. All rats were allowed to access to food and drinks *ad libitum* when they were not under training.

3.4.3.1 Voluntary wheel running

The V-Ex rats were housed individually in a cage with a running wheel assembly and let free to run (manufactured by Kan Kee Sheet Metal Works, Hong Kong). The circumference of the wheel was 1 meter, and the wheel was connected to a switch that counted the number of revolutions (Fig 3.14a) (Hayes et al 2008). The

running distance of the V-Ex rats was recorded and collated on a daily basis. The daily average running distance of the V-Ex group rats is shown in figure 3.14b.



(a) (b)

Figure 3.14. The wheel cage system for the V-Ex group rats (a) and the average daily running distance of the V-Ex group rats (b). The white arrow indicates the counter which counts the number of revolutions of the rats, and the number shown in the counter represents the running distance of the V-Ex rats. (b) The quantity of exercise undertaken by the V-Ex rats was represented by their average running distance. The vertical bars in the graph represent the standard deviations (SD), while I1 to I7 represent the days of intervention after surgery.

3.4.3.2 Forced treadmill running

The F-Ex rats were forced to run on the motor-driven treadmill (KN-73, Natsume Ltd., China) at a speed of 20m/min with a slope of 0° for a total of 30 minutes every day (Fig 3.15) (Yang et al 2003; Nomura et al 2005). The 30-minute exercise was divided to three 10-minute sessions and with a 10-minute rest between successive running sessions. The rats would receive a nudge on the back when they did not catch up with the treadmill (Li et al 2009). The running duration on the treadmill was based on a pilot experiment on rat wheel running and the experiment setup was intended to match the running distance between V-Ex and F-Ex. In this study, the distances were similar between the V-Ex and the F-Ex

groups (on average 622.33 m and 600 m, respectively) on the first day of intervention (I1), and the V-Ex rats increased running distance during their intervention period (Fig 3.14b).

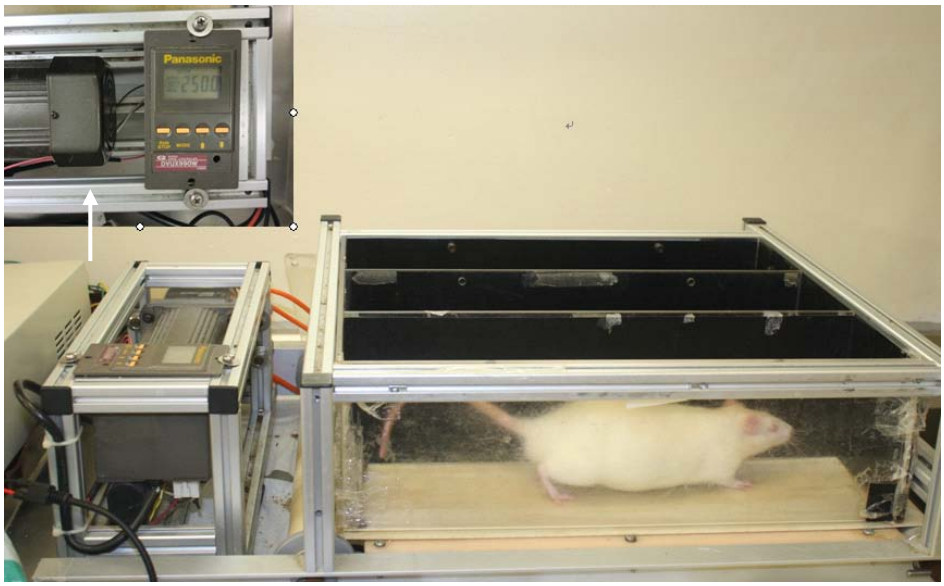


Figure 3.15. The treadmill system for the F-Ex group rats. The F-Ex group rats were forced to run on a treadmill at a speed of 20 m/min for totally 30 minutes per day. The while arrow indicates the control panel which adjusts the running speed of the treadmill.

3.4.3.3 Involuntary functional electrical stimulation (FES) training

The I-Ex group rats received a total of 30 minutes FES every day. The involuntary exercise of FES (I-Ex) with implanted electrodes in two hind limb muscles on the affected side was to mimic gait-like walking pattern during stimulation (Gillis and Biewener 2001). The stimulation pulse was a biphasic rectangular pulse with a frequency 100 Hz, pulse width 300 μs and stimulation intensity was based on the muscle threshold for contraction during anesthesia (Leung et al 2007). The 30 minutes stimulation was divided to three 10-minute sessions and with a 10-minute rest between each session. The training and the rest periods in the stimulation protocol were planned to match with those of the F-Ex

intervention protocol. The stimulation pattern (50 ms TA stimulation, 150 ms MG stimulation, and 300 ms rest) imitated the normal gait-like pattern of rat running on the treadmill at the speed of 20 m/min (Fig 3.16).

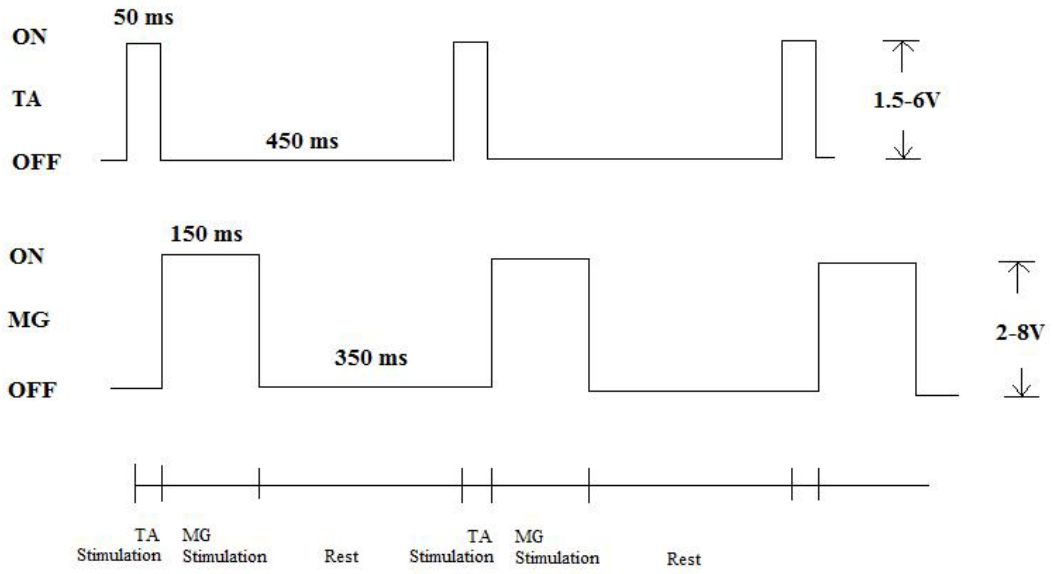


Figure 3.16. Stimulation pattern generated by the pattern generator. This stimulation pattern mimics the gait-like pattern during running at a speed of 20m/min for rats.

3.4.4 Evaluation Tools

After the MCAo/r surgery, the rats' body weight and behavioral scores were measured and recorded on a daily basis throughout the 7-day intervention period. The brain BDNF concentrations and serum corticosterone levels were evaluated after the sacrifice of the rat after the intervention period.

3.4.4.1 Motor Function Recovery Test

In this study, the De Ryck's behavioral test, which had been validated in the pilot study, was applied to evaluate the motor function recovery after stroke as it

appeared to be particularly sensitive in detecting impairments and deficits after brain ischemia caused by MCAo (Belayev et al 1996). The behavioral test was conducted daily during the 7-day intervention period (I1-I7) and repeated for three times after everyday intervention and the average score was recorded. This behavioral test consists of 8 subtests which were discussed in previous section 3.2.3, and it was to examine sensorimotor integration in forelimb placing responses to visual, tactile and proprioceptive stimuli. Figure 3.17 shows the evaluation process of each subtest.

Figure 3.17. De Ryck's behavioral tests of rat after right MCAo/r surgery. (a) Test 1: affected limb (circled) did not touch the ground when the rat was descent from a higher position; (b) test 2: forelimb on the affected side (circled) could not place on the table edge when the head was tilted; (c) test 3: affected limb (circled) could not place on the table edge simultaneously as unaffected limb when the rat faced the table edge; (d) test 4 (forelimb & hindlimb): affected hand placed on the table edge when the rat was placed near edge after recovery; (e) test 5 (forelimb & hindlimb): affected limbs (circled) did not place on the table edge when the rat was pushed laterally to the table edge; and (f) test 6: affected limb (circled) could not grasp the table edge when the head was placed near the table edge.

3.4.4.2 Beam Walking Test

This test, developed by Schallert et al, involves scoring foot slips during transverse of a 2 cm wide * 130 cm long wooden beam (Schallert et al 2002). The beam was elevated 110 cm from the ground and placed horizontally with the home cage placed at the target end. The rats were placed at the one end of the beam, and their foot slips were counted during their walk to their home cage. The test was performed each day throughout the 7-day intervention, and the averaged score of 3 transverses was recorded. The maximal score is 6, meaning that there was no foot slips during the transverse, and the minimal is 0, meaning that the rat could not maintain a balance on the beam. The detailed scoring system of beam walking test was described in Table 2.7.

3.4.4.3 Brain BDNF and Serum Corticosterone Evaluation

Brain BDNF concentrations and serum corticosterone level were determined with commercial ELISA kits as ELISA is a sensitive and common tool for detecting these chemicals in rat tissues (Hayes et al 2008). All rats were sacrificed by decapitation under anesthesia within 2 hours of their last intervention and assessment. Trunk blood was collected immediately after decapitation and centrifuged to obtain the serum for corticosterone level determination by ELISA kit (Assay Design, USA). The brain infarction volumes were measured by TTC staining method, and it was shown that cortex and striatum were affected after MCAo (Fig 3.13). After sacrifice, the rat's brain was obtained by removing the

skull, and the cerebral cortex was carefully removed to extract the striatum and hippocampus. The striatum, the motor cortex of the affected area, and the hippocampus were obtained to assess the levels of BDNF by E_{max}® ELISA kit (Promega, Wisconsin, USA). All procedures were carried out according to the manufacturer's instructions.

3.4.5 Statistical Analysis

Results were expressed as means ± SD. SPSS (version 15.0) was used for statistical analyses in this study. One-way analysis of variance (ANOVA) with Bonferroni post-hoc test was used to compare the serum corticosterone level and the BDNF levels in the hippocampus, striatum, and cortex among four groups. In the comparison based on the behavioral test and body weight, multivariate analysis of covariance (MANCOVA) incorporating all outcome measures recorded from I1-I7 was used to reduce the probability of type I error owing to multiple comparisons (Tabachnick and Fidell 1996). This is a technique for assessing group differences across multiple metric-dependent variables simultaneously, based on a set of categorical variables as independent variables. The within-subject factor was set as time and the between-subject factor was set as group. The I1 measurement of each respective outcome was entered as covariate. If the MANCOVA revealed a significant effect, post-hoc analysis using Bonferroni correction was used to indicate significant differences between particular groups. The level of statistical significance was set at 0.05.

CHAPTER 4

RESULTS

4.1 Study I: Pilot Study for Animal Stroke Models Setup

The pilot study was conducted to determine the duration of cerebral artery occlusion and the duration of exercise intervention and to testify the ELISA technique in BDNF detection.

4.1.1 Accommodation and MCAo/r Surgery

60 young male Sprague-Dawley (SD) rats with body weights 280-320g were used in the pilot study. 9 rats were dropped out after the 3-day accommodation period as they did not meet the minimal requirements (averaged 600 m/day in running distance in wheel cage) of the selection criteria (drop out rat around 15%). The remaining 51 rats were randomly divided into three groups for different occlusion time in the middle cerebral artery occlusion/reperfusion (MCAo/r) surgery: 60 minutes (n=17), 90 minutes (n=17), and 120 minutes (n=17). The mortality and success rate for each group was shown in Table 4.1. It was found that the overall success rate was highest in the 90-min occlusion group. Therefore, 90-min occlusion was determined as a suitable duration to produce considerable survival rate with ischemic stroke. This protocol was used in the whole study.

Table 4.1 Occlusion duration vs. mortality/success rate (data were approximated from pilot study)

	60 minutes (n=17)	**90 minutes (n=17)**	**120 minutes (n=17)**
Mortality	20%	30%	60%
Percentage of rats with strokes	40%	70%	80%
Overall success rate	30%	50%	30%

4.1.2 BDNF Level

The brain-derived neurotrophic factor (BDNF) concentrations in lumber and hippocampus were evaluated in the three groups: baseline group (B, n=9), one-week group (G1, n=9), and two-week group (G2, n=9). Figure 4.1 shows the BDNF levels in lumber and hippocampus regions in these three groups. Significant differences were found between G1 and B groups ($p<0.0001$), and between G2 and B groups ($p<0.0001$). The results indicated that the lumbar BDNF level was not sensitive compared with the hippocampal BDNF level, and there was no significant difference between one-week and two-week interventions in their effects on BDNF levels. As a result, a one-week exercise intervention was regarded sufficient in affecting the hippocampal BDNF level, and the lumbar BDNF level detection was ceased in further study.

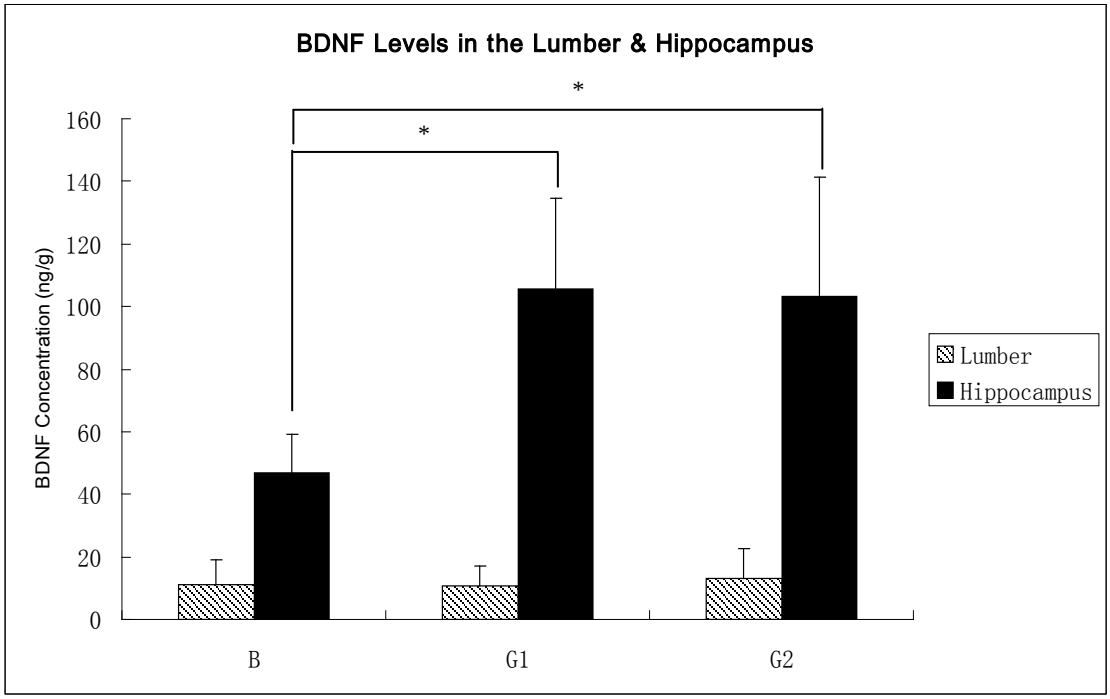


Figure 4.1. The lumbar & hippocampal BDNF levels. *: significant differences were found between B (n=9) & G1 (n=9) groups, and B & G2 (n=9) groups using SPSS one-way ANOVA with Bonferroni post-hoc analysis.

4.1.3 Investigation of Ischemic Stroke Model and Hemorrhagic Stroke Model

This study was to investigate the cerebral blood flow changes, the brain damages and the neurological deficits in ischemic stroke model and hemorrhagic stroke model. The experiment of ischemic stroke part was conducted by Dr. Li Le and the author together, and the data was analyzed independently. The experiment of hemorrhagic stroke part was conducted and the results were analyzed by the author completely. In this study, 40 young male Sprague-Dawley rats (3 months) were used. 12 rats were excluded in this study, among which 5 rats did not show motor deficits after MCAo/r surgery, and 7 rats died within 24 hours after MCAo/r surgery. A total of 28 stroke rats with motor deficits were assigned into two groups:

Group	n
Ischemic stroke	14
Hemorrhagic stroke	14

All the rats in two groups finished the 4-day experiment, and their body weight, behavioral score, and cerebral BFV were measured daily throughout the experiment. Table 4.2 shows the overall results of cerebral BFV changes along the experiment period.

Table 4.2 Summery of Time-averaged Maximum BFV in Ischemic Stroke Group (n=14) and Hemorrhagic Stroke Group (n=14) Rats

Time-averaged maximum BFV (cm/s)		**Ischemic stroke**	**Hemorrhagic stroke**
RMCA	Baseline	11.8 ± 2.2	16.5 ± 3.0
	D0	5.8 ± 1.4	9.9 ± 2.7
	D1	8.5 ± 1.2	10.3 ± 2.5
	D2	8.0 ± 1.6	11.5 ± 3.4
	D3	7.5 ± 1.9	12.0 ± 3.1
	D4	8.4 ± 1.3	13.1 ± 4.3
LMCA	Baseline	14.3 ± 4.1	14.0 ± 4.8
	D0	10.7 ± 4.7	11.9 ± 4.2
	D1	13.9 ± 5.3	11.1 ± 2.3
	D2	13.1 ± 6.0	10.7 ± 2.5
	D3	14.3 ± 6.6	11.1 ± 2.8
	D4	13.7 ± 6.4	11.2 ± 2.4
RICA	Baseline	58.9 ± 14.3	55.6 ± 9.8
	D0	- 32.8 ± 18.6	49.5 ± 11.5
	D1	-43.9 ± 16.5	48.9 ± 7.1
	D2	-55.9 ± 19.0	47.5 ± 12.6
	D3	-43.3 ± 15.4	62.1 ± 13.0
	D4	-47.7 ± 12.2	64.1 ± 19.2
LICA	Baseline	53.9 ± 12.9	55.7 ± 8.3
	D0	61.1 ± 12.9	53.5 ± 14.7
	D1	74.6 ± 12.6	59.9 ± 11.9
	D2	76.1 ± 11.3	51.0 ± 10.2
	D3	86.7 ± 16.3	52.5 ± 13.2
	D4	81.5 ± 13.8	59.6 ± 13.9
BA	Baseline	17.7 ± 2.3	16.6 ± 2.5
	D0	20.5 ± 6.6	14.8 ± 2.1
	D1	29.8 ± 5.2	16.3 ± 3.6
	D2	31.6 ± 5.3	14.8 ± 3.0
	D3	33.1 ± 8.3	18.4 ± 4.6

	D4	31.0 ± 8.5	16.6 ± 1.6
RPCA	Baseline		16.0 ± 2.1
	D0		11.9 ± 2.8
	D1		14.4 ± 4.5
	D2	-	14.1 ± 3.1
	D3		18.1 ± 5.0
	D4		17.3 ± 2.4
LPCA	Baseline		14.7 ± 4.3
	D0		13.3 ± 3.1
	D1		15.1 ± 3.7
	D2	-	11.3 ± 6.8
	D3		19.5 ± 6.7
	D4		17.4 ± 5.1

Values are expressed as mean ± SD; D0 represents 30 minutes after the surgery, whereas D1 means 1 day after the surgery. "–"sign indicated a reversed blood flow was detected in the target artery (Li L, Ke Z, Tong KY, and Ying M 2010).

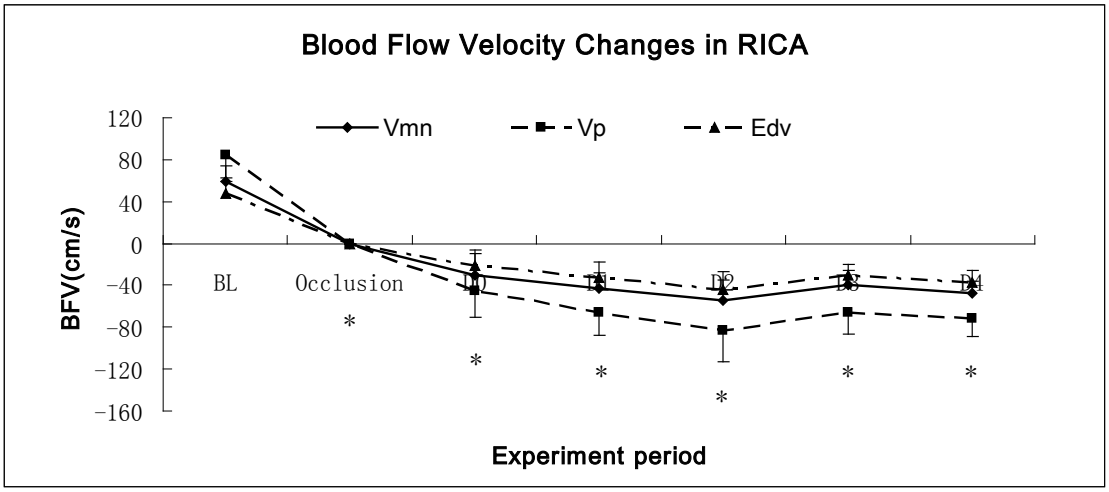
4.1.3.1 Reliability Test

The ICC reliability test resulted in a range of 0.876 to 0.992 of Cronbach α for all seven arteries (Li L, Ke Z, Tong KY, and Ying M 2010), indicating a good inter-rater reliability (Meyers and Blesh 1962). The test-retest reliability resulted in a range of 0.925 to 0.988 of Cronbach α for two operators (Li L, Ke Z, Tong KY, and Ying M 2010), indicating high intra-rater reliability (Meyers and Blesh 1962).

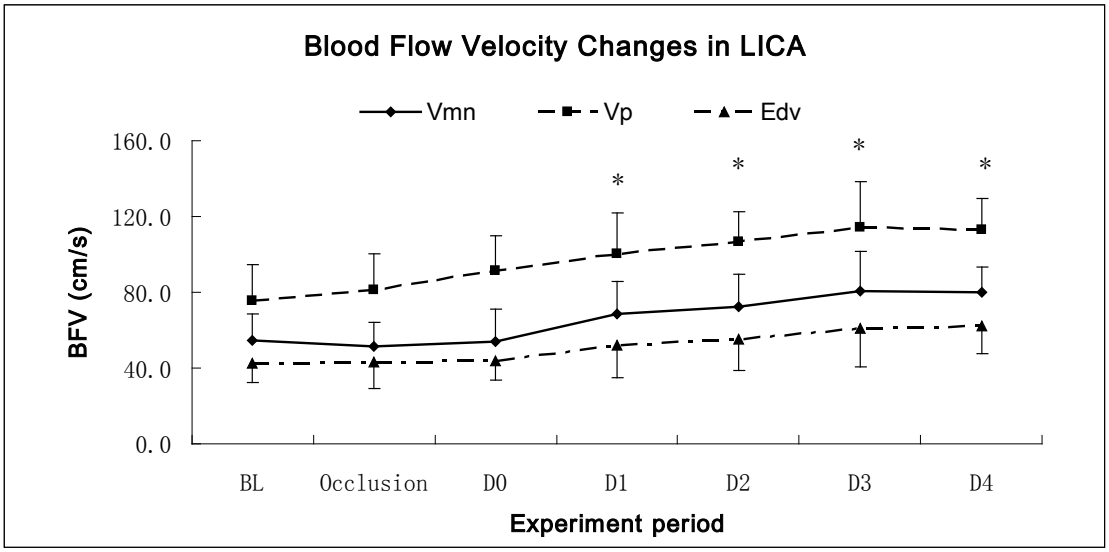
4.1.3.2 BFV Changes in Ischemic Stroke Rats

The blood flow velocity changes in RICA, LICA, RMCA, LMCA, and BA are shown in figure 4.2a, b, c, d, e, respectively. Data are expressed as mean ± SD. Significant differences were found in RICA, LICA, RMCA, and BA. Post-hoc test revealed the significant differences between baseline (BL) value and other

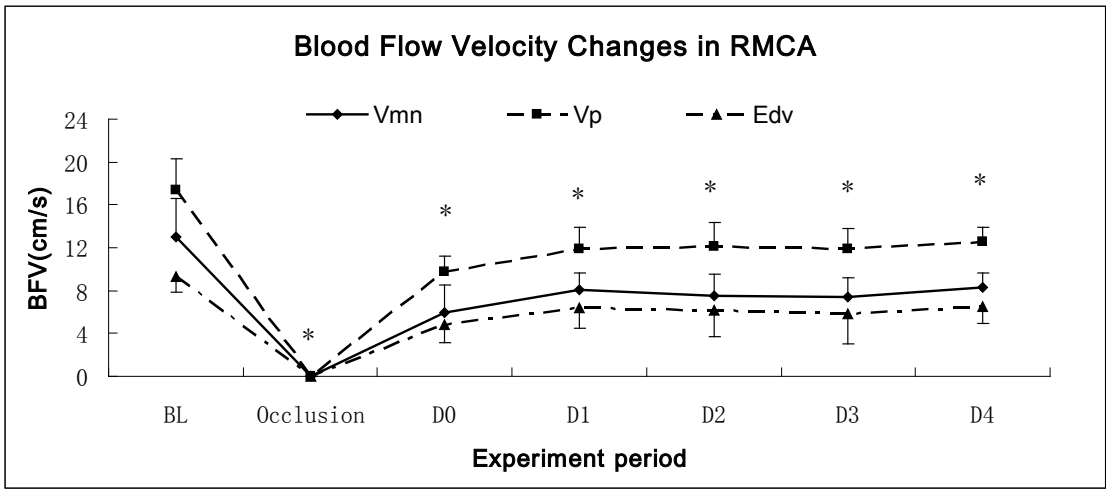
time points along the experiment period. Briefly, the BFV in RICA and RMCA significantly decreased from the time 'occlusion' during the MCAo/r surgery, and this change lasted throughout the experiment. On the other hand, the BFV in LICA and BA significantly increased 24 hours after the surgery, and lasted throughout the experiment as well. The BFV in LMCA remained in a steady state and did not show any significant changes (Li L, Ke Z, Tong KY, and Ying M 2010).



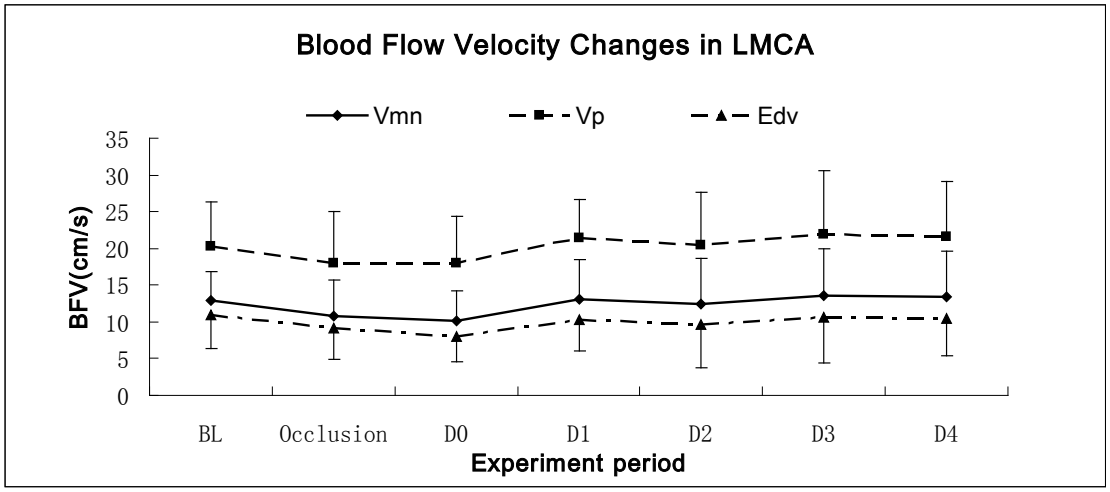
(a)



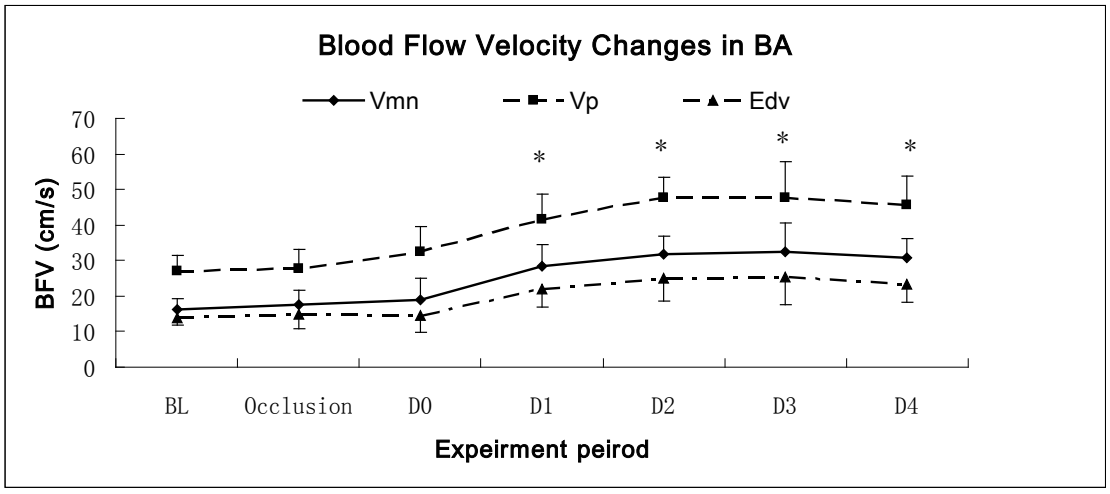
(b)



(c)



(d)



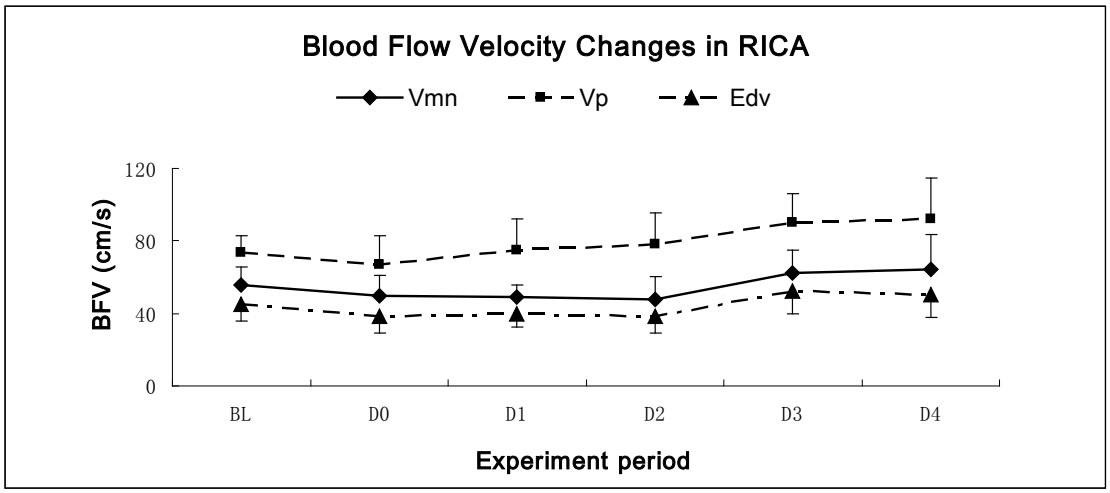
(e)

Figure 4.2. The BFV changes in RICA, LICA, RMCA, LMCA, and BA in ischemic stroke rats (n=14). BL represents baseline, whereas D0 represents 30 mins after the MCAo/r surgery, and D1

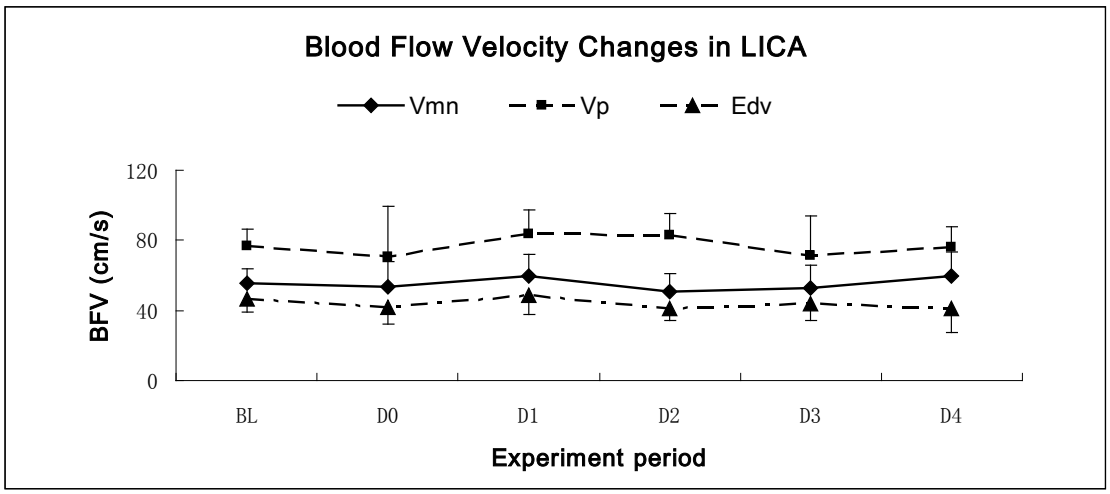
means 24 hours after the surgery, and ect.. (a) blood flow dropped to zero in RICA during the occlusion as two terminals (RCCA & RMCA) were blocked; blood flow changed its direction after reperfusion (D0) as the blockage at distal end (RMCA) was released while the proximal end (RCCA) was permanent ligated. (b) BFV in LICA gradually increased along the experiment, and increased to a significant level from D1-D4. (c) BFV dropped to zero in RMCA during the occlusion as RMCA was blocked by a filament, and it raised to certain level after reperfusion, but was still significantly lower than the baseline level throughout the experiment; (d) BFV in LMCA did not show significant change throughout the experiment; (e) similar as in LICA, BFV in BA gradually increased along the experiment, and increased to a significant level from D1-D4. * Any significant difference between the baseline and other time of time-average maximum (Vmn) BFV ($p<0.05$). The error bar represents 1 standard deviation (SD) (Li L, Ke Z, Tong KY, and Ying M 2010).

4.1.3.3 BFV Changes in Hemorrhagic Stroke Rats

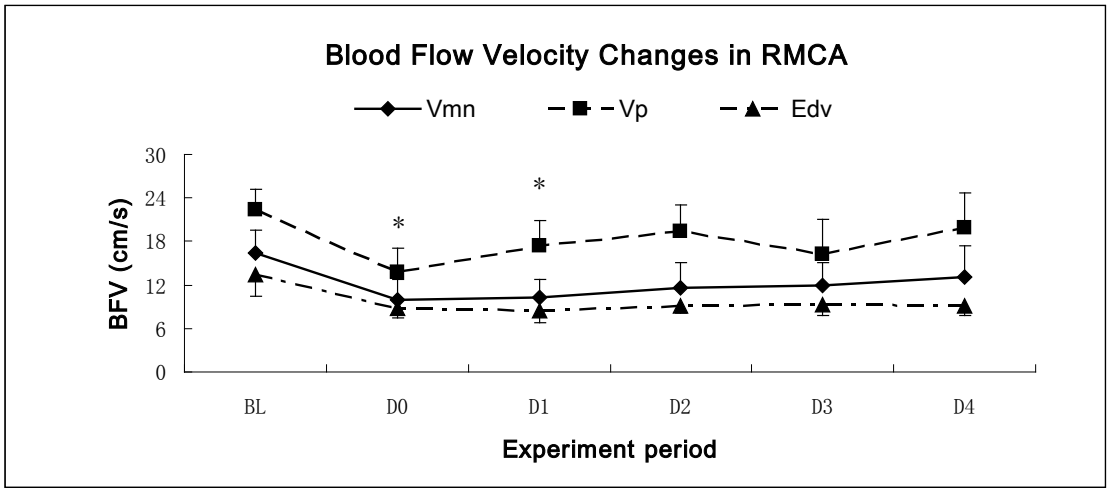
The blood flow velocity changes in RICA, LICA, RMCA, LMCA, BA, RPCA, and LPCA are shown in figure 4.3a, b, c, d, e, f, g, respectively. Data are expressed as mean +/- SD. Significant differences were found in RMCA and RPCA. Post-hoc test revealed the significant differences between baseline (BL) value and other time points along the experiment period. Briefly, the BFV in RMCA and RPCA significantly decreased from baseline data 30 minutes after the ICH surgery. The decrease of BFV in RMCA was also found at 24 hour point (D1), but not in RPCA and other time points. No significant differences were found in bilateral ICA, LMCA, BA, or LPCA.


Blood Flow Velocity Changes in RICA
Vmn
Vp
Edv
BFV (cm/s)
120
80
40
0
BL
D0
D1
D2
D3
D4
Experiment period

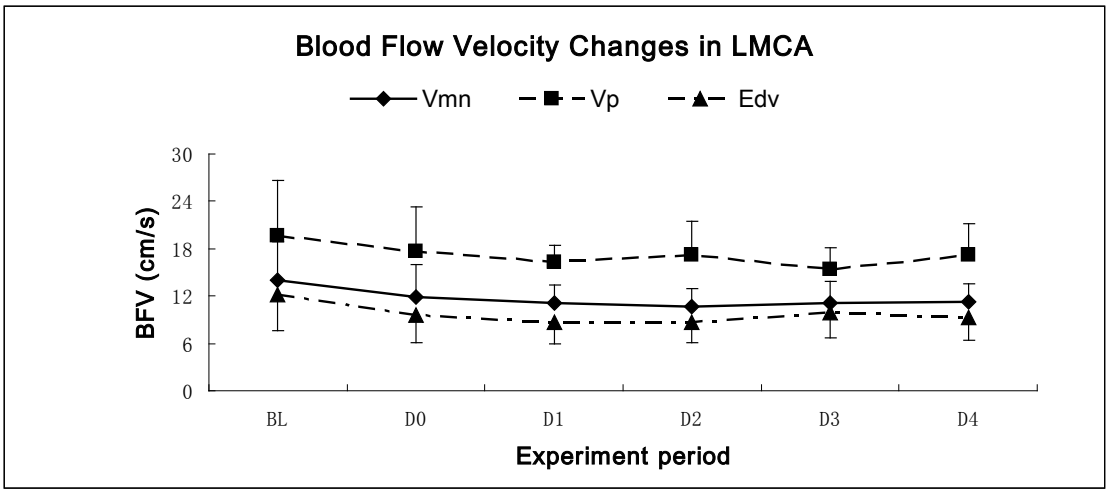
(a)


Blood Flow Velocity Changes in LICA
Vmn
Vp
Edv
BFV (cm/s)
120
80
40
0
BL
D0
D1
D2
D3
D4
Experiment period

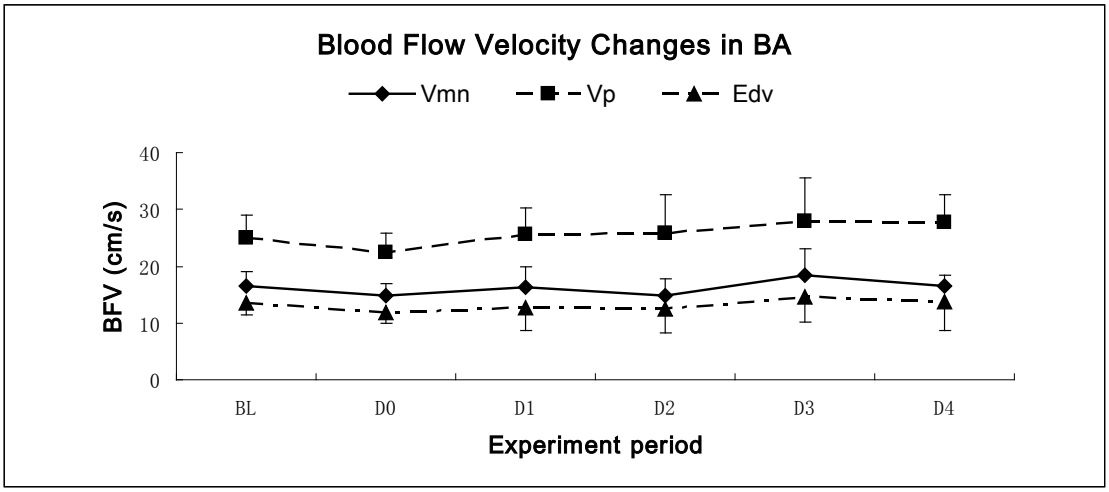
(b)


Blood Flow Velocity Changes in RMCA
Vmn
Vp
Edv
BFV (cm/s)
30
24
18
12
6
0
*
*
BL
D0
D1
D2
D3
D4
Experiment period

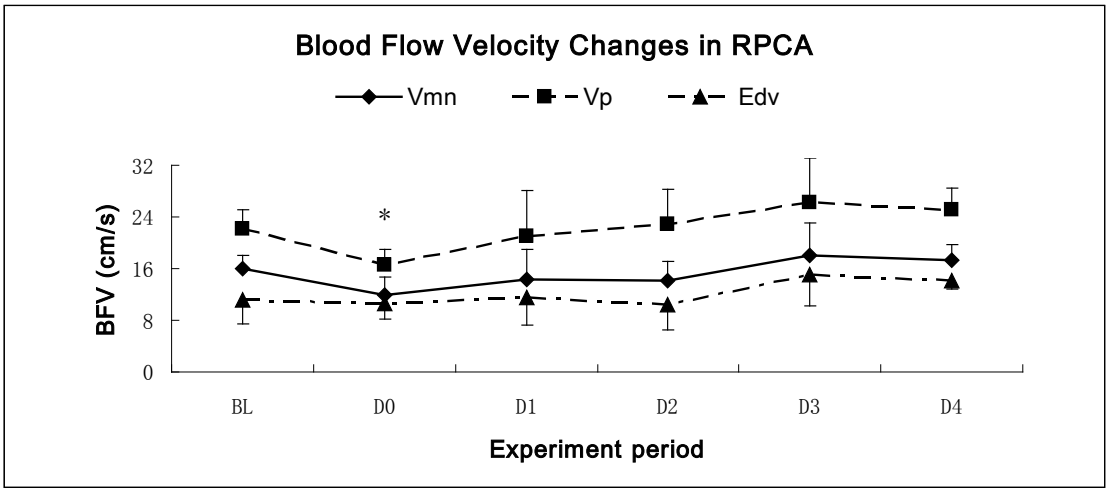
(c)


Blood Flow Velocity Changes in LMCA
Vmn
Vp
Edv
BFV (cm/s)
30
24
18
12
6
0
BL
D0
D1
D2
D3
D4
Experiment period

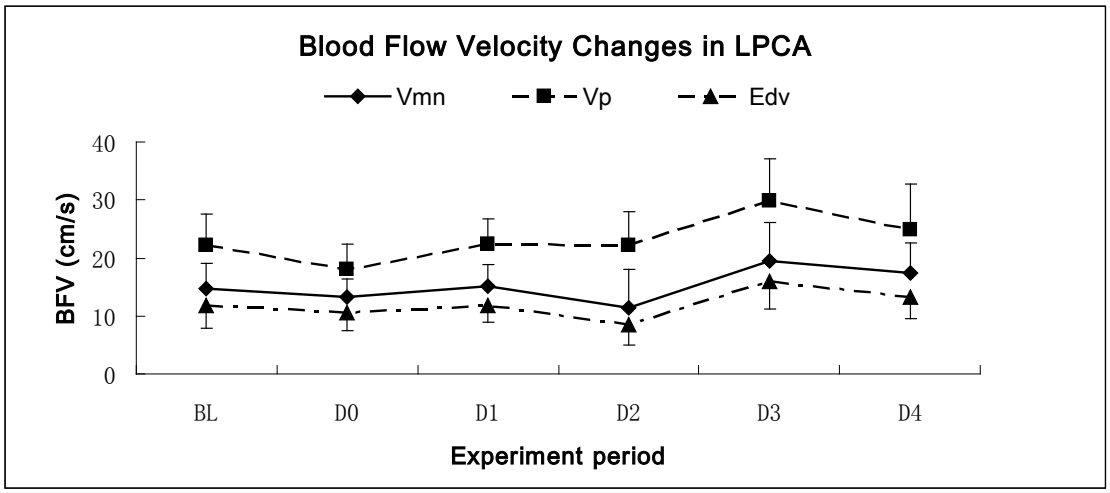
(d)


Blood Flow Velocity Changes in BA
Vmn
Vp
Edv
BFV (cm/s)
40
30
20
10
0
BL
D0
D1
D2
D3
D4
Experiment period

(e)


Blood Flow Velocity Changes in RPCA
Vmn
Vp
Edv
BFV (cm/s)
32
24
16
8
0
*
BL
D0
D1
D2
D3
D4
Experiment period

(f)

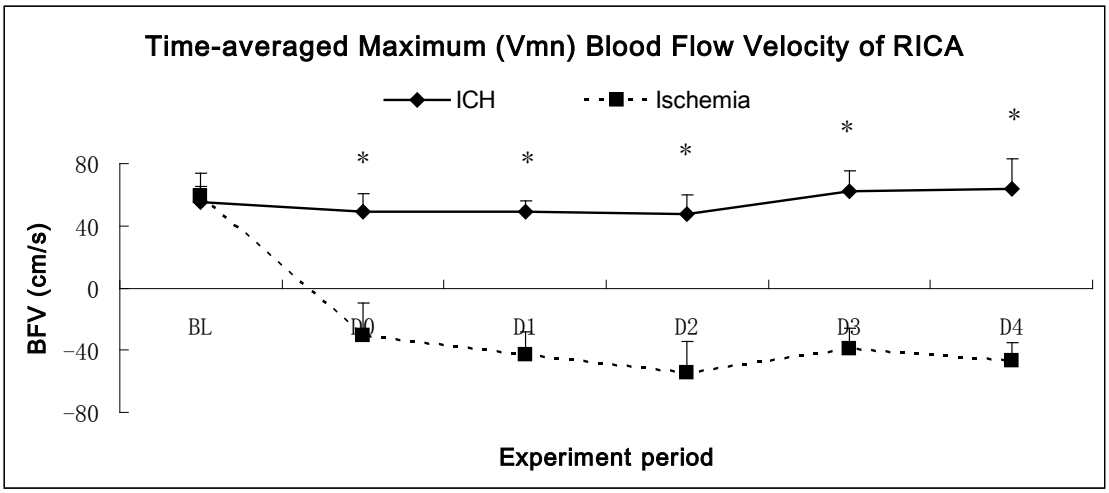


(g)

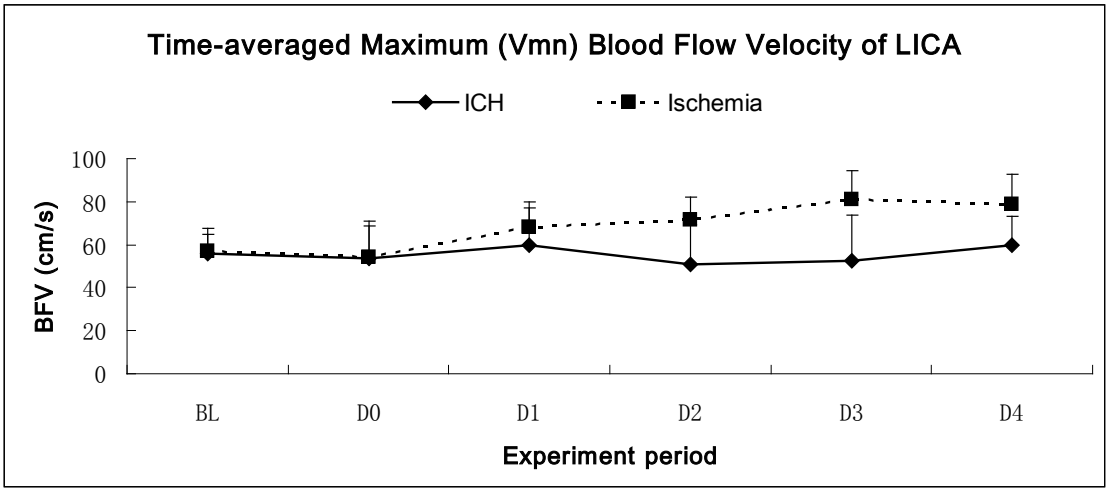
Figure 4.3. Blood flow velocity changes before and after the ICH surgery and along the post-stroke recovery stage of (a) RICA, (b) LICA, (c) RMCA, (d) LMCA, (e) BA, (f) RPCA, and (g) LPCA in hemorrhagic stroke rats (n=14). Significant differences were found between the baseline and other time points of time-average maximum (Vmn) BFV at RMCA and RPCA. *: $p<0.05$. The error bar represents 1 standard deviation (SD).

4.1.3.4 The BFV changes between Ischemic Stroke Rats & Hemorrhagic Stroke Rats

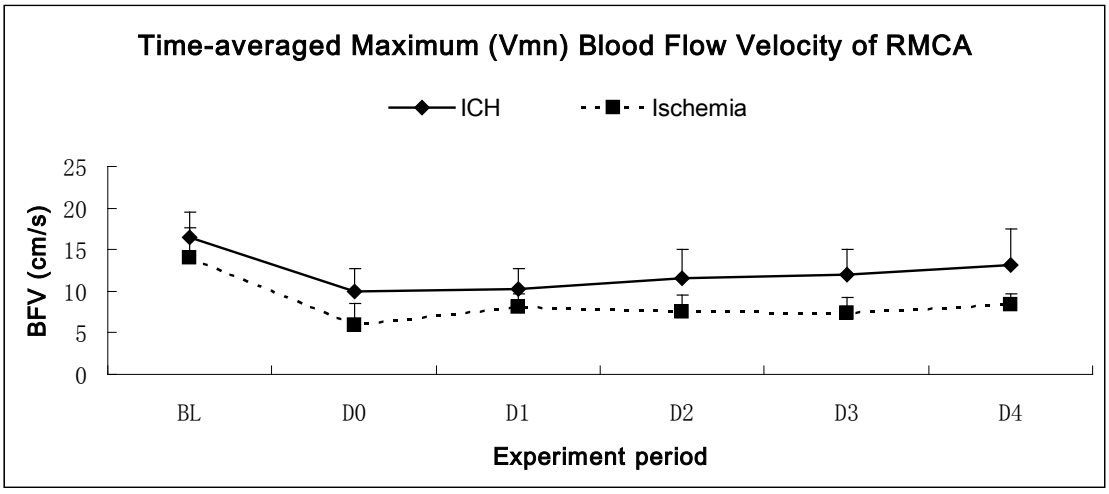
The time-averaged maximum (Vmn) BFV in ischemic stroke and hemorrhagic stroke groups were compared using MANCOVA analysis in five arteries, which are RICA, LICA, RMCA, LMCA, and BA. Significant differences were found in the BFV of RICA and BA. The BFV of RICA in hemorrhagic stroke group was significantly higher than that in ischemic stroke group immediately (around 0.5 hours) after the ICH or MCAo/r surgery, as there was a reversed blood flow in RICA in after reperfusion in the MCAo/r surgery. The BFV of BA, however, in ischemic stroke group was significantly higher than that in hemorrhagic stroke group from 24 hours after the surgeries (Li L, Ke Z, Tong KY, and Ying M 2010).


Time-averaged Maximum (Vmn) Blood Flow Velocity of RICA
ICH
Ischemia
BFV (cm/s)
80
40
0
−40
−80
BL
D0
D1
D2
D3
D4
*
Experiment period

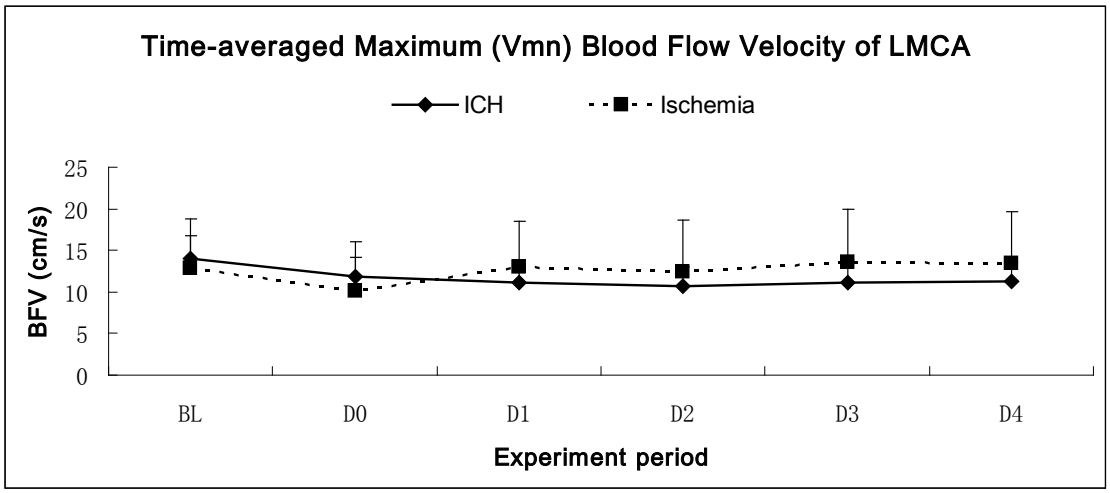
(a)


Time-averaged Maximum (Vmn) Blood Flow Velocity of LICA
ICH
Ischemia
BFV (cm/s)
100
80
60
40
20
0
BL
D0
D1
D2
D3
D4
Experiment period

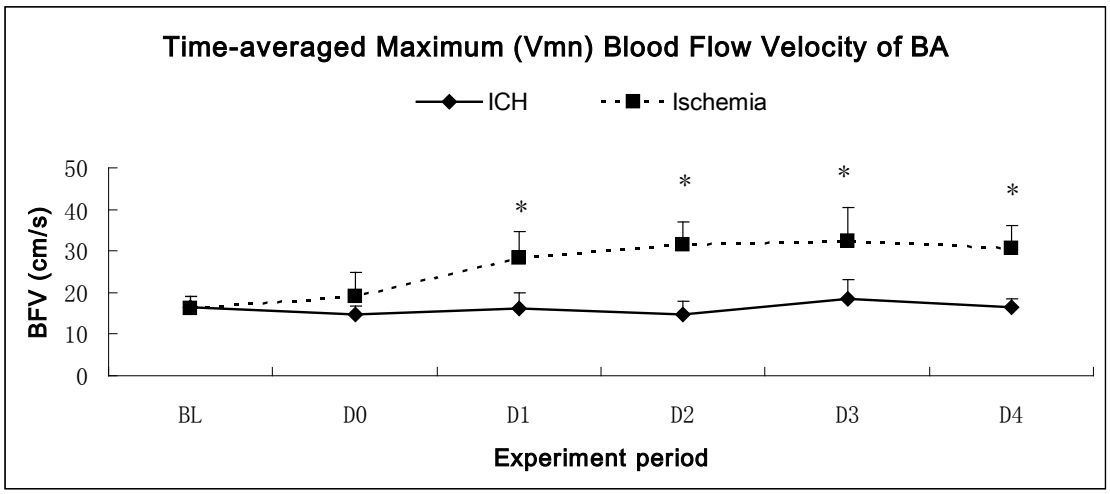
(b)


Time-averaged Maximum (Vmn) Blood Flow Velocity of RMCA
ICH
Ischemia
BFV (cm/s)
25
20
15
10
5
0
BL
D0
D1
D2
D3
D4
Experiment period

(c)



(d)



(e)

Figure 4.4. Comparisons of time-averaged maximum (Vmn) BFV changes between ischemic stroke group (n=14) and hemorrhagic stroke group (n=14) rats in (a) RICA, (b) LICA, (c) RMCA, (d) LMCA, and (e) BA. Significant differences were found in RICA and BA. *: $p<0.05$. The error bar represents 1 standard deviation (SD) (Li L, Ke Z, Tong KY, and Ying M 2010).

4.1.3.5 Heart Rate

The heart rate changes in two group rats are shown in figure 4.5. On the basal state, heart rate of ischemic stroke group and hemorrhagic stroke group rats were 428 ± 25 and 418 ± 31 beats per minute (bpm), respectively. Neither MCAo/r surgery nor ICH surgery affect the heart rate (411 ± 27 bpm for ischemic stroke and 409 ± 30 for hemorrhagic stroke). During the post-stroke stage, the heart rate

ranged from 394 ± 45 bpm to 419 ± 48 bpm for ischemic stroke, and 382 ± 37 bpm to 422 ± 42 bpm for hemorrhagic stroke. No significant difference was found along the time points or between two groups.

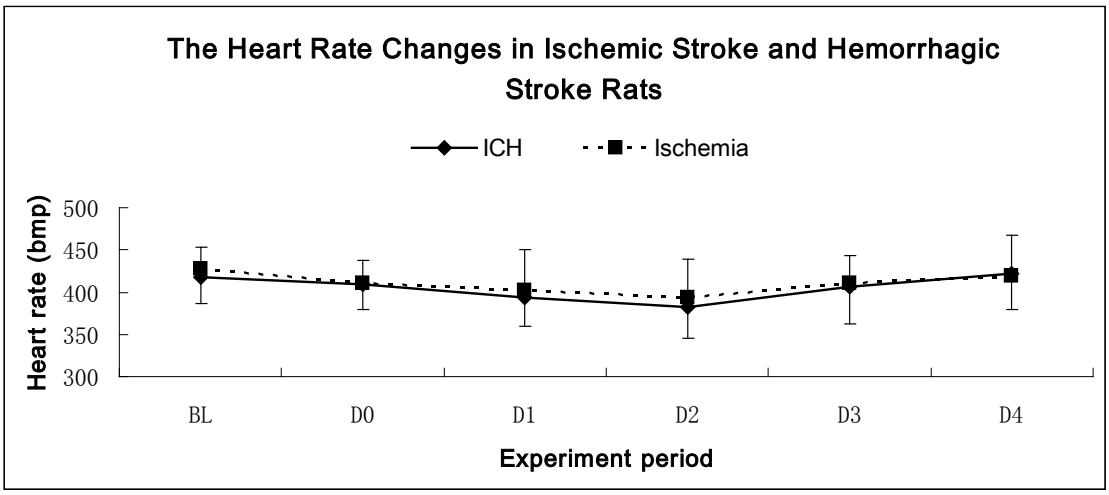


Figure 4.5. The changes of heart rate in two group (n=14 in each group) rats along the experimental period. No significant difference was found at different time points during the ultrasonic assessments.

4.1.3.6 Body Weight

The body weight changes in two group rats are shown in figure 4.6. The average body weight of both ischemic stroke group and hemorrhagic stroke group rats did not show significant change after the MCAo/r and ICH surgeries, and kept relatively stable throughout the experiment period (Li L, Ke Z, Tong KY, and Ying M 2010).

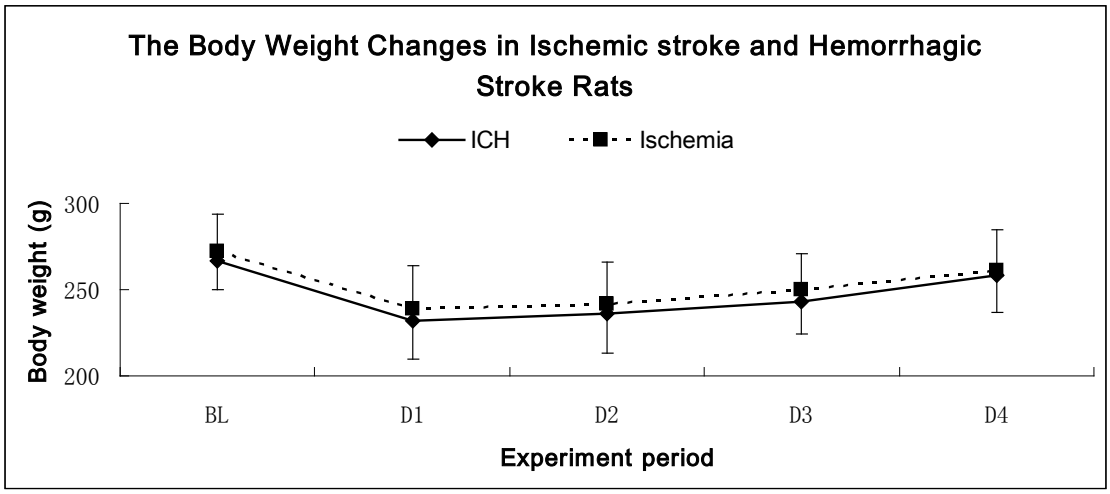


Figure 4.6. The changes of body weight in two group (n=14 in each group) rats along the experimental period. Although there was a drop of body weight after the MCAo/r and ICH surgeries, no significant difference was found at different time points throughout the experiment (Li L, Ke Z, Tong KY, and Ying M 2010).

4.1.3.7 De Ryck's Behavioral Test Scores

The averaged daily behavioral test scores in two group rats are shown in figure 4.7. Rats showed motor deficit after the MCAo/r or ICH surgery and had a low behavioral test score when first examined (D1). In ischemic stroke group, the behavioral test score increased significantly from D2-D4 after the MCAo/r surgery compared with D1 after the surgery, whereas such significant increase started from D3 in hemorrhagic stroke group. No significant difference, however, was found between two groups (Li L, Ke Z, Tong KY, and Ying M 2010).

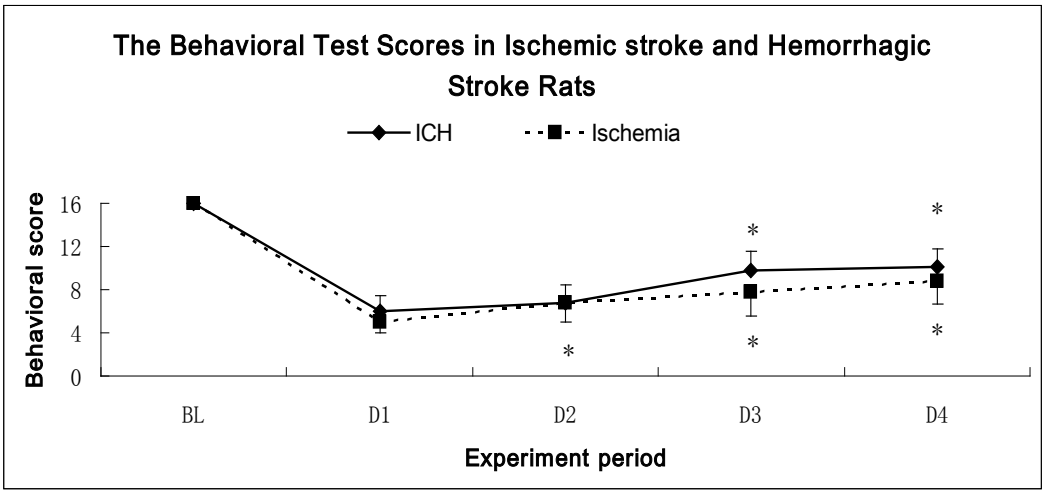
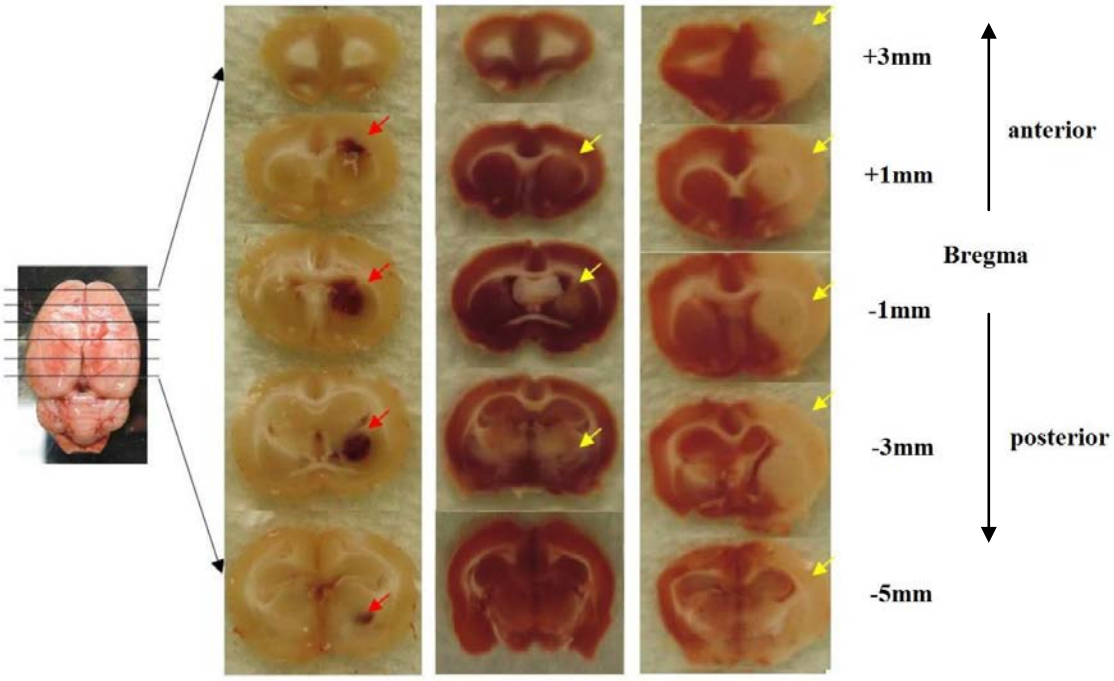


Figure 4.7. Behavioral tests results in two group (n=14 in each group) rats on a daily measured basis. Significant differences were found between post 24 h (D1) and other time points in the behavioral score within the same group. *: $p<0.05$ (Li L, Ke Z, Tong KY, and Ying M 2010).

4.1.3.8 Brain Infarct Volume Measured by TTC Staining

The averaged ischemic volume and hematoma volume of two group rats measured on TTC-stained histological sections were 148 ± 56 mm^3 and 6.8 ± 0.7 mm^3, respectively (Li L, Ke Z, Tong KY, and Ying M 2010). The ischemic stroke and hemorrhagic stroke brain slices were shown and compared figure 4.8.



(a) (b) (c) (d)

Figure 4.8. Brain slices imaging for comparison of the hemorrhagic stroke and the ischemic stroke. TTC reacts with dehydrogenases in viable cells and results in a "brick-red" color, and the white area indicates the infarction. (a) The rat brain was sliced into 2 mm sections; (b) brain slice without TTC staining 24 hours after ICH surgery (D1), red arrow indicates hematoma (c) brain infarction was revealed by TTC staining 4 days after ICH surgery, yellow arrow indicates infarction; (d) brain infarction was revealed by TTC staining 4 days after ischemia stroke, yellow arrow indicates infarction.

4.2 Study II: Comparison of the Effects of Different Exercise Interventions as Stroke Rehabilitation Tools in a Rat Cerebral Ischemia Model

This study was to compare the effectiveness of voluntary exercise, involuntary exercise, and forced exercise as stroke rehabilitation interventions. All the experiments were performed by the author and the results have been published on journal PLoS One (Ke Z, Yip SP, Li L, Zheng XX, Tong KY (2011) The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor

and motor function recovery: a rat brain ischemia model. PLoS One 6(2):e16643). 150 young male Sprague-Dawley rats (3 months) were used in this experiment. 93 rats were excluded in this study, among which 33 rats did not pass the accommodation, 24 rats did not show motor deficits after MCAo/r surgery, and 36 rats died within 24 hours after MCAo/r surgery. The overall success rate was similar or even higher in our experiment when compared with other studies (Longa et al 1989; Kitagawa et al 1998; Petullo et al 1999). A total of 57 ischemic stroke rats with motor deficits were assigned into four groups:

Group	n
Voluntary wheel exercise group (V-Ex)	14
Involuntary FES exercise group (I-Ex)	14
Forced treadmill exercise group (F-Ex)	15
Control group (Con)	14

45 of these rats finished the 7-day intervention. Twelve rats that died during the intervention period were not included in the BDNF and corticosterone analysis, and the intention-to-treat method was used to analyze their beam-walking test and behavioral test scores, which means that the scores of their unfinished days were the same as that of the last score that we recorded on the day before it died. Table 4.2 shows the overall results of the behavioral test, the serum corticosterone concentration, and the brain BDNF levels in the hippocampus, cortex, and striatum. Significant differences were revealed in all the tests.

Table 4.3 Comparison of Motor Function Recovery, Brain BDNF Levels, and Stress Response Outcome among four group rats

Variable	Group	Pre-intervention	Post-intervention	*P*	Post hoc(*P*)
Behavioral Score	V-Ex	4.70±1.16	11.90±1.20	<0.0001*	V-Ex&I-Ex(0.014*) V-Ex&F-Ex(<0.0001*) V-Ex&Con(<0.0001*) I-Ex&F-Ex(0.808) I-Ex&Con(0.351) F-Ex&Con(1.000)
	I-Ex	5.18±1.33	10.54±1.97		
	F-Ex	5.63±1.03	9.73±1.56		
	Con	4.00±0.82	7.90±1.29		
Beam Walking Score	V-Ex	1.81±0.52	5.72±1.27	<0.0001*	V-Ex&I-Ex(1.000) V-Ex&F-Ex(0.001*) V-Ex&Con(0.001*) I-Ex&F-Ex(0.035*) I-Ex&Con(0.056) F-Ex&Con(1.000)
	I-Ex	1.95±0.57	5.59±1.82		
	F-Ex	1.67±0.25	4.44±1.26		
	Con	1.33±0.34	3.17±0.75		
Hippocampal BDNF Level(ng/g)	V-Ex	-	96.48±29.36	<0.0001*	V-Ex&I-Ex(0.652) V-Ex&F-Ex(<0.0001*) V-Ex&Con(<0.0001*) I-Ex&F-Ex(<0.0001*) I-Ex&Con(0.008*) Con&F-Ex(0.015*)
	I-Ex		83.05±13.90		
	F-Ex		18.29±5.92		
	Con		51.78±6.08		
Striatal BDNF Level(ng/g)	V-Ex	-	25.10±10.39	0.005*	V-Ex&I-Ex(0.461) V-Ex&F-Ex(0.478) V-Ex&Con(0.857) I-Ex&F-Ex(0.007*) I-Ex&Con(0.019*) Con&F-Ex(1.000)
	I-Ex		35.32±7.93		
	F-Ex		14.96±5.09		
	Con		16.45±9.78		
Cortical BENF Level(ng/g)	V-Ex	-	17.40±8.93	0.035*	V-Ex&I-Ex(1.000) V-Ex&F-Ex(0.584) V-Ex&Con(1.000) I-Ex&F-Ex(0.053) I-Ex&Con(0.141) Con&F-Ex(1.000)
	I-Ex		21.00±8.02		
	F-Ex		12.15±4.88		
	Con		13.23±7.43		

					F-Ex&V-Ex(<0.0001*)
Serum	V-Ex		223.71±73.79		F-Ex&I-Ex(<0.0001*)
Corticosterone	I-Ex	-	394.72±83.70	<0.0001*	F-Ex&Con(<0.0001*)
Concentration	F-Ex		656.51±156.57		I-Ex&Con(0.001*)
(nmol/l)	Con		211.13±47.17		I-Ex&V-Ex(0.001*)
					Con&F-Ex(1.000)

Values: mean±SD; *P* value: significance level of MANCOVA multiple comparisons with covariates for beam-waling score and behavioral score; significance level of one-way ANOVA with Bonferroni post-hoc analysis for BDNF levels and corticosterone concentration.

*Indicates significant differences were revealed; post hoc was performed to specify the effect of group difference.

4.2.1 Body Weight

The body weight of individual rat was measured every morning before the behavioral test assessment. MANCOVA was used as the statistical analysis tool to reduce the probability of type I error owing to multiple comparisons. No significant difference was found among groups.

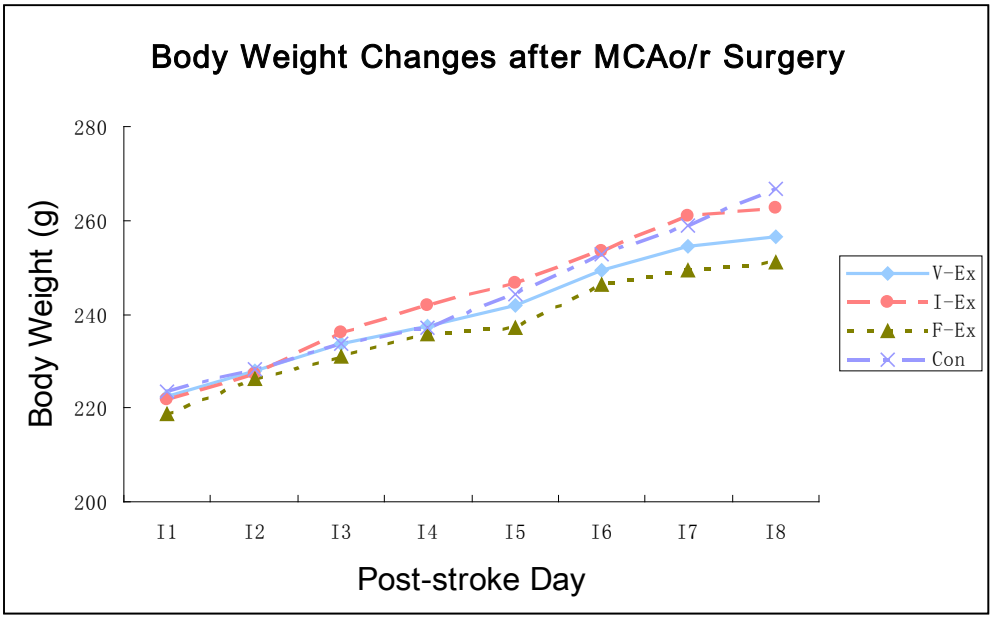


Figure 4.9. The daily body weight changes after MCAo/r surgery among four groups.

4.2.2 De Ryck's Behavioral Test Score

The motor function recovery outcome was presented as the behavioral score change and is shown in Figure 4.10. All group rats gradually increased the behavioral scores along the intervention period (I1-I7). Rats undertaken different interventions, however, showed different degrees of recovery. Significant differences among the four groups were found from day I3 and lasted until the end of the intervention period in the behavioral score (MANCOVA $p<0.0001$). In the post-hoc test, the V-Ex had significant higher behavioral test score than the I-Ex ($p=0.014$), the F-Ex ($p<0.0001$), and the Con ($p<0.0001$) at the end of the intervention (I7). Significant differences were not found between other groups at I7.

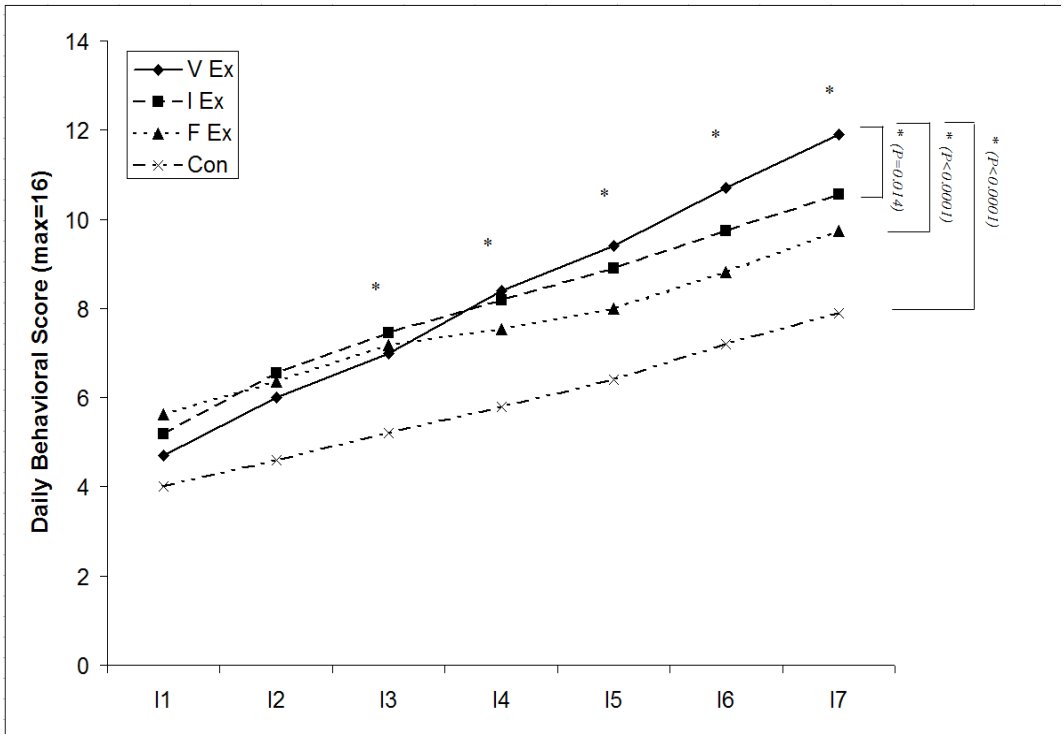


Figure 4.10. The daily behavioral score changes of four group rats during intervention period. The motor recovery is evaluated with daily behavioral score of the V-Ex (♦), I-Ex (■), F-Ex (Δ), and Con (x) during the 7-day intervention. *:a significant difference is revealed with MANCOVA from I3, and at the end of the training period V-Ex has significant higher

behavioral score than I-Ex, F-Ex and Con in the post-hoc, indicating that voluntary exercise is more effective in facilitating motor recovery than involuntary exercise, forced exercise, and spontaneous recovery. Significant difference is not found between other groups after the 7-day intervention.

4.2.3 Beam Walking Test Score

The daily beam walking score change is shown in figure 4.11. Significant differences among the four groups were found in the last-day beam walking score (MANCOVA *P<0.0001*). Both the V-Ex and the I-Ex groups perform better than the F-Ex and the Con groups in the beam walking test.

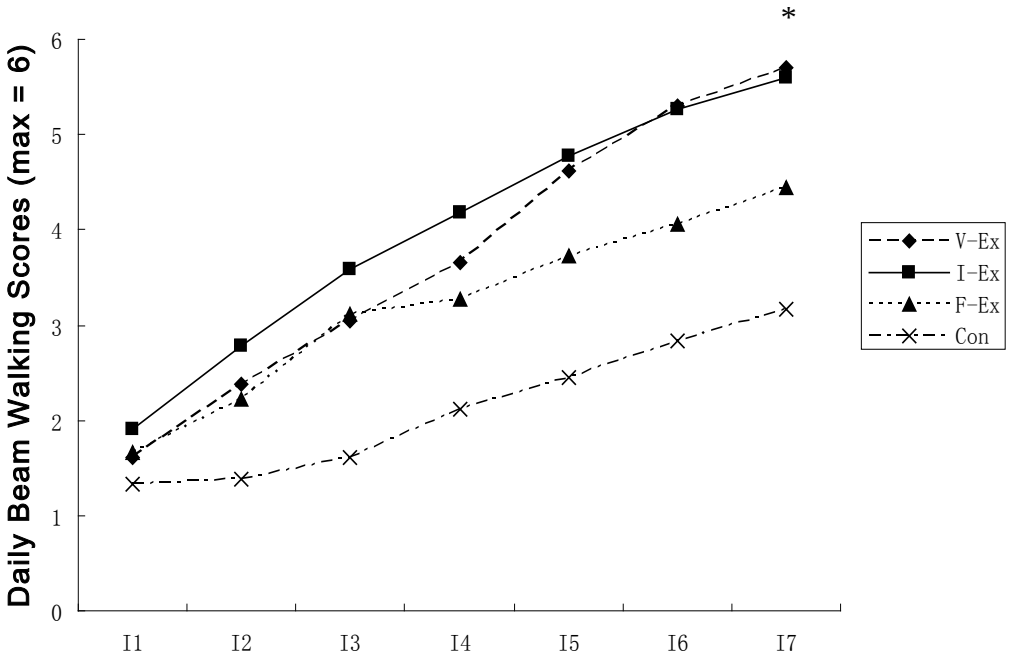


Figure 4.11. The daily beam walking score of the V-Ex (♦), I-Ex (■), F-Ex (Δ), and Con (x) during the 7-day intervention. *:a significant difference was revealed with MANCOVA (V-Ex has significant difference with F-Ex and Con in the post-hoc test for beam walking test).

4.2.4 Brain BDNF Levels

The brain BDNF levels were detected in hippocampus, cortex, and striatum. The results were analyzed with one-way ANOVA and values were presented as mean

± SD. Significant differences are revealed with one-way ANOVA.

Figure 4.12a shows the comparison of BDNF levels among four groups. Both the V-Ex and the I-Ex groups had higher hippocampal BDNF concentration than the F-Ex and the Con groups: V-Ex vs. F-Ex ($p<0.0001$); V-Ex vs. Con ($p<0.0001$); I-Ex vs. F-Ex ($p<0.0001$); I-Ex vs. Con ($p=0.008$). Besides, the I-Ex group had significantly higher striatal and cortical BDNF concentrations than the F-Ex and the Con groups: I-Ex vs. F-Ex ($p=0.007$); I-Ex vs. Con ($p=0.019$).

Figure 4.12b shows the comparison of BDNF levels among different areas (hippocampus, striatum, and cortex) of the brain. In the V-Ex, I-Ex, and Con groups, hippocampal BDNF levels are significantly higher than both striatal BDNF levels ($p<0.0001$ in the V-Ex, I-Ex, and Con groups) and cortical BDNF levels ($p<0.0001$ in the V-Ex, I-Ex, and Con groups). In the F-Ex group, no significant differences were found among hippocampal, striatal, and cortical BDNF levels.

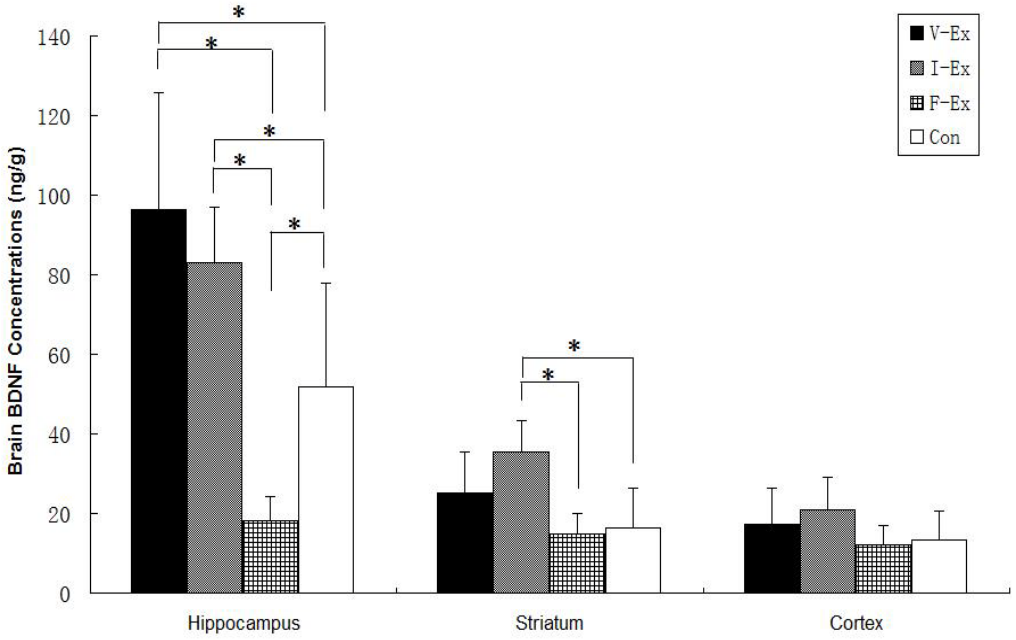


Figure 4.12a. The brain BDNF level comparison among four groups. Values shown are mean ± SD *: a significant difference was revealed with one-way ANOVA with Bonferroni post-hoc analysis. The V-Ex (n=13) and I-Ex (n=11) groups had higher hippocampal BDNF concentration than the F-Ex (n=11) and Con (n=10) groups. The I-Ex group also had higher striatal BDNF concentration than the F-Ex and Con groups.

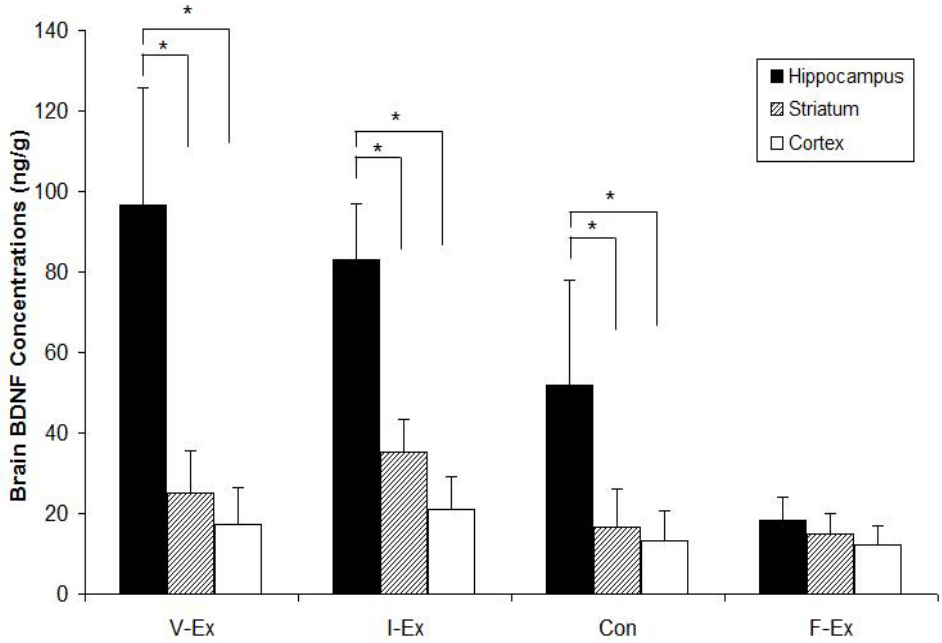


Figure 4.12b. The brain BDNF level comparison among different areas of the brain. Values shown are mean ± SD *: a significant difference was revealed with one-way ANOVA with Bonferroni post-hoc analysis. In the V-Ex, I-Ex, and Con groups, hippocampal BDNF levels are significantly higher than striatal and cortical BDNF levels.

4.2.5 Serum Corticosterone Concentration

The serum corticosterone concentration is shown in Figure 4.13. Results were analyzed with one-way ANOVA, and values were presents as presented as mean ± SD. The F-Ex group exceeded other three groups in the serum corticosterone concentration: F-Ex vs. V-Ex ($p<0.0001$), F-Ex vs. I-Ex ($p<0.0001$), F-Ex vs. Con ($p<0.0001$). The average corticosterone concentration of the F-Ex group was around 3 times of those of the V-Ex group and the Con group (656.51 nmol/l for F-Ex group, 223.71 nmol/l for V-Ex group, and 211.13 nmol/l for Con group). The I-Ex group also had significantly higher serum corticosterone concentration than the V-Ex and Con groups: I-Ex vs. V-Ex ($p=0.001$), I-Ex vs. Con ($p=0.001$).

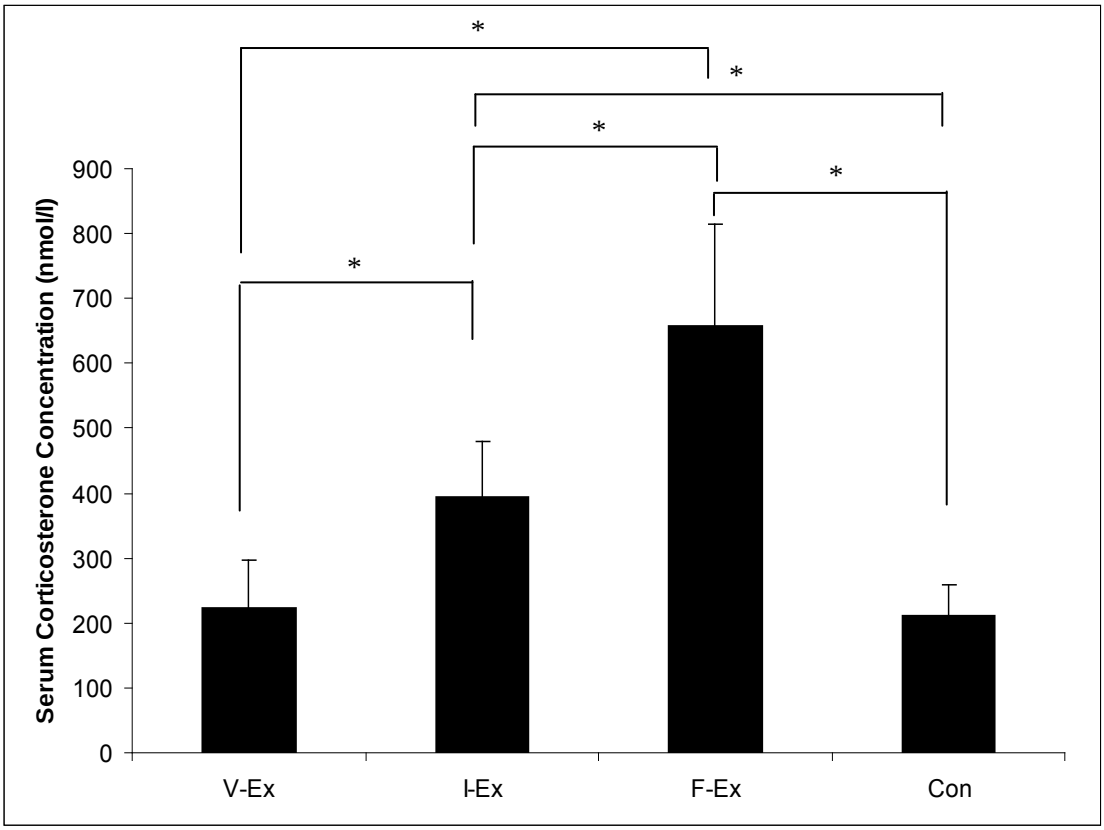


Figure 4.13. The serum corticosterone levels among four groups. Values shown are mean ± SD *: a significant difference was revealed with one-way ANOVA with Bonferroni post-hoc analysis. The F-Ex group (n=11) had significantly higher serum corticosterone level than other three groups, followed by the I-Ex group (n=11). Both the V-Ex (n=13) and the Con (n=10) groups had

relatively low serum corticosterone level.

CHAPTER 5

DISCUSSION

This study firstly investigated the cerebral blood flow changes, the brain damage and the neurological deficits in the ischemic stroke model and the hemorrhagic stroke model. Comparisons were then conducted to study the effectiveness of voluntary wheel exercise, involuntary functional electrical stimulation (FES) exercise, and forced treadmill exercise on brain-derived neurotrophic factor (BDNF) regulation and motor recovery levels using the ischemic stroke model. In this chapter, the cerebrovascular and physiological changes induced by middle cerebral occlusion/reperfusion (MCAo/r) and intracerebral hemorrhage were firstly discussed. The effects of different exercise regimens on facilitating motor recovery after ischemic stroke were then examined based on the results presented in Chapter 4. A discussion on the neurochemical changes mediated by these exercise interventions was followed. Finally, possible mechanisms of recovery under different types of exercise training were suggested.

5.1 Investigations of Cerebral Blood Flow Changes, Brain Damage, and Neurological Deficits in Ischemic and Hemorrhagic Stroke Models

5.1.1 Focal Cerebral Ischemia with Intraluminal Suture MCAo/r

Intraluminal suture occlusion of MCA has become the standard laboratory model of focal cerebral ischemia in rats (O'Neill and Clemens 2001). With this method, the relationship between occlusion durations and brain damage is well characterized (Schilinhting et al. 2004). The occlusion period of intraluminal suture model for producing infarction varied from 45 minutes to 3 hours in published reports (Connoly et al. 1996; Bruced et al. 1996; Hara et al. 1996). It was shown that, however, the occlusion time less than 60 minutes could hardly produce significant infarct, whereas occlusion period equaled to or longer than 180 minutes effectively represented a permanent occlusion and caused massive infarcts. As a result, in our pilot study, occlusion periods were set from 60 minutes to 120 minutes. Our results shown that the mortality rate and the percentage of the rats that obtained strokes were proportional to the occlusion time (Table 4.1), indicating that the longer occlusion time is, the more likely that rats can obtain strokes and the more severe brain damage is produced. This result is in accordance with previous studies that less than 50% rats had infarcts with 60 minutes MCAo, and infarct size increased as the duration of MCAo increased (Fig 5.1) (Belayev et al. 1996; Schilinhting et al. 2004). Failure of inducing stroke by intraluminal suture model can be explained by the variation of vessel structure and morphology, the pattern of blood supply in MCA by collateral arteries and most importantly, the time of occlusion.

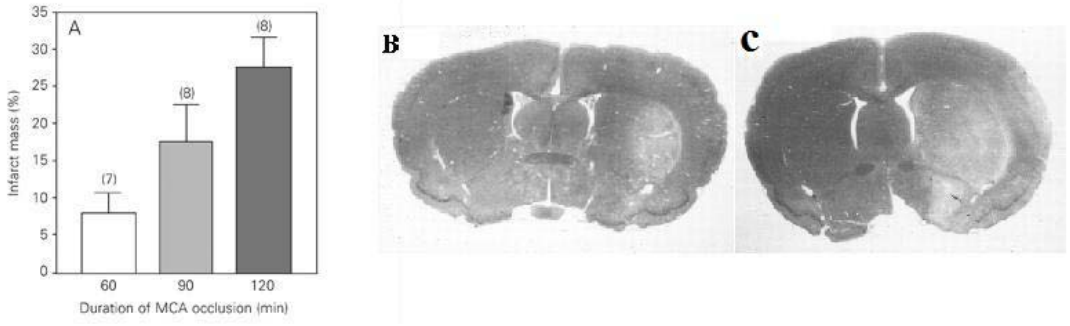


Figure 5.1. Duration of MCA occlusion vs brain damage. (A) Occlusions of MCA for 60 min, 90 min, and 120 min result in a proportional increased infarct size; (B) Occlusion of MCA for 60 minutes only result in a small portion of infarct at ipsilateral striatum; (C) Occlusion of MCA for 120 minutes result in an extensive infarct involves dorsolateral and lateral portions of the cortex. (from Schilinhting et al. 2004; Belayev et al. 1996).

Longer duration of MCA occlusion ensures the neurological deficits in rats. It also results in, however, an increased mortality rate. In our results, the mortality rate of 60 min, 90 min, and 120 min MCA occlusion were 20%, 30%, and 60% respectively, suggesting that the current technique could effectively block the blood flow to the MCA region and cause considerable damage to the brain. This is consistent with the published mortality rate using the same intraluminal suture model (Longa et al. 1989; Nagasawa and Kogure 1989; Kitagawa et al. 1998; Connolly et al. 1996; Petullo et al. 1999). In Petullo et al., the mortality rate with 120 min MCA occlusion was the same as our result (Petullo et al. 1999), while in another study, mortality rate with only 60 min MCA occlusion could reached 80% (Kitagawa et al. 1998). Rats died after MCA occlusion could be attributed to different reasons. In some cases, hemorrhage was observed along the cerebral vessel network upon dissection, and it was probably caused by excessive insertion pressure from the intraluminal suture (Garcia 1993). In other cases, massive infarcts were produced and caused irreversible brain damage and lethal

neuron deaths, devastating the regulation and metabolism systems and eventually resulted in death (Harukuni 2006).

Considering the percentage of rats obtaining cerebral ischemia as well as the mortality rate, we also calculated the overall success rate, which was the percentage of rats that survived with cerebral ischemia, under different durations of MCA occlusion. The result turned out that 90 min occlusion was preferred as it produced consistent and repeatable deficits with acceptable mortality rate (Table 4.1). As a result, the 90-min occlusion protocol was used in the following experiments.

5.1.2 Intracerebral Hemorrhage with Collagenase Infusion

The intrastriatal injection of collagenase method, which effectively disrupts the basal lamina of cerebral capillaries and causes bleeding in the brain parenchyma, was developed to produce intracerebral hemorrhage and a reproducible hematoma (Rosenberg et al. 1990). This model was a reliable and consistent method that mimicked the human hemorrhagic stroke, and the amount of collagenase ranged from 0.014 units to 0.35 units were able to produce clear ICH deterioration in striatum areas (Terai et al. 2003). In accordance with this statement, our results showed that the successful rate of inducing hemorrhagic stroke with 0.23 units collagenase infusion was 100% (14 out of 14 rats were induced ICH), and the hemorrhage was found in striatum areas as well (Fig 4.8). In addition, the brain infarct was localized at the areas where hemorrhage was found from the TTC

staining (Fig 4.8), which was also consistent with previous literature that bleeding amount and hematoma size were linearly correlated (Terai et al. 2003). Finally, it was found that the TTC staining was not sensitive enough in infarct volume detection in the current ICH model (section 4.2.7), which was also reported in the study of Patel's group (Patel et al.. 1999). This limitation was due to the original purpose of current study to investigate both the ischemic stroke and the hemorrhagic stroke models with same evaluation method, and should be implemented in future studies.

5.1.3 Reliability Test of Ultrasonography Techniques

In this study, cerebral blood flow velocity (BFV) of ischemia stroke rats and hemorrhagic stroke rats along the stroke-recovery process was evaluated through ultrasonography. Previous studies have substantiated the feasibility of ultrasonography application in assessment of cerebral blood flow velocity in small animals (Els et al.. 1999; Bonnin et al. 2008). Our result of reliability test has also shown good inter-rater (0.876 to 0.992) and high intra-rater (0.925 to 0.988) reliability with the same ultrasonography technique (section 4.2.1). The following scales for indicating levels of reliability were applied: equal or above 0.90, high reliability; 0.80 to 0.89, good reliability; 0.70 to 0.79, fair reliability; and 0.69 or less, poor reliability (Meyers and Blesh 1962).

5.1.4 Investigations of Cerebral Blood Flow Changes between Ischemic Stroke Rats and Hemorrhagic Stroke Rats

The cerebral blood flow changes were evaluated as the BFV changes in five major cerebral arteries: RICA, LICA, RMCA, LMCA, and BA. MANCOVA revealed significant differences in the BFV of the RICA and the BA between ischemic stroke rats and ICH rats.

The BFV of the RICA in hemorrhagic stroke group was significantly higher than that in ischemic stroke group immediately (around 0.5 hours) after the ICH or MCAo/r surgery, as there was a reversed blood flow in the RICA in after reperfusion in the MCAo/r surgery (Fig 4.4a). This finding was also documented in a previous study on brain hypoxia and ischemia of rat pups, in which a reversed blood flow was found in the left ICA when this artery was persistently occluded (Bonnie et al. 2008). This phenomenon was explained by the collateral blood flow adaptation through the Willis circle to maintain and the restoration of the blood flow supply in the occluded artery. It is known that the anterior and posterior circulation is connected via the circle of Willis, which is formed by the unpaired ACA and the paired posterior communication artery (Fig 2.4). In the ischemic stroke model, as the RCCA and the RECA are permanently occluded by ligation, the reversed flow in the RICA is properly caused by the contralateral blood supply through the circle of Willis, which is well acknowledged in human circulation (Hendrikse et al. 2001). In addition, it is shown that rats share the similarity with human that they also permit collateral blood flow through the

circle of Willis (Small and Buchan 2000). On the other hand, the BFV in the RICA in ICH rats did not change significantly along the stroke-recovery state, indicating that the current ICH model leave the RICA intact. This phenomenon can result from two aspects: (1) the surgery procedure did not involve operation on the RICA; (2) the hemorrhage did not affect the artery which locates away from the bleeding region.

Another difference between ischemic rats and ICH rats was the BFV change in the BA (Fig 4.4e). From 24 hours to 96 hours after the stroke onset, BFV in the BA of ischemic stroke rats was significantly higher than that of ICH rats. This result can be attributed to the hyper-perfusion of cerebral blood flow after brain ischemia. Post-ischemia hyper-perfusion is a common situation in human stroke, and was also shown in rats (Marchal et al. 1999; Nakamura et al. 2008). Upon the occlusion of the RICA, blood supply can be retrieved immediately from BA under a compensation mechanism, which is directly connected to RICA in anatomical structure (Fig 2.4). Besides, collateral blood supply that is infused in to the RICA and its branches can also through the circle of Willis, to which the BA served as one of the major original supplies. As a result, BFV in the BA increased gradually after MCA reperfusion to restore blood supply and maintain the cerebral steadiness (Fig 4.2e).

In both cerebral ischemia and ICH experiments, a significantly decreased BFV in RMCA after the onset of stroke was observed (Fig 4.2c, 4.3c, 4.4c). In the ischemic stroke model, the BFV of RMCA decreased to 0cm/min during

occlusion, supporting that the current technique can prevent the blood flow to the MCA territory completely. After reperfusion in the RMCA, the BFV remained in a decreased level when it was compared with baseline value (Fig 4.2c). This was in accordance with the previous animal studies in which decreased cerebral blood flow in the ipsilateral MCA post-surgery was revealed (Els et al. 1999; Ding et al. 2002). Since the compensated blood supply of ischemia area comes from the collateral blood vessels, it is reasonable that the velocity is slower than the original supply, which is directly from the main branch of the ICA. Moreover, it may also be the reason that BFVs of arteries on the affected side were smaller than those on the unaffected side after cerebral ischemia. On the other hand, the rapid decrease of BFV in the RMCA was also observed in the ICH model (Fig 4.3c). One possible explanation is that hemorrhage induces hypoxia in the bleeding area, which further reduces cerebral blood flow in the perihematomal zone. The collagenase-induced ICH model applied in current study produces lesions in the dorsolateral and middle regions of the striatum (Kirik et al. 1998), and these regions are mainly supplied by middle cerebral artery and posterior cerebral artery (Bederson et al. 1986; Takagi et al. 1995). Previous studies also showed similar results. In rat ICH model, cerebral blood flow was reduced in bleeding areas after hemorrhage occurred (Yang et al. 1994). Clinically, Zazulia and colleagues demonstrated a reduction of the oxygen metabolism rate and oxygen extraction in the perihematomal zone in 19 ICH patients, indicating hypoxia occurred, which resulted in a local deceased cerebral blood flow (Zazulia

et al. 2001).

Other differences of BFV changes between ischemic stroke model and ICH stroke model were shown in the LICA and RPCA. Although there was no significant difference in BFV changes in the LICA between two models (Fig 4.4b), BFV in the LICA was significantly increased in ischemia stroke rats but not in ICH rats when they were compared with baseline value. The increased BFV in the LICA may also be due to the blood supply compensation mechanism: when the extracranial RICA was ligated, blood flow from contralateral size had to increase to keep the brain in a steady state. While in ICH model, both sides of ICA were intact. BFV in the RPCA (Fig 4.3f), which was not measured in ischemic stroke rats, was significantly dropped immediately after the ICH surgery but returned to a normal level 24 hours later. These differences, which were uncharted in previous literature, may suggest that during ischemia, the whole cerebral vascular circulation is disturbed, whereas during hemorrhage, only arteries supplying the hemorrhagic tissues are affected.

5.1.5 Heart Rate, Body Weight, Behavioral Score, and Brain Staining with TTC

In both ischemic stroke rats and ICH stroke rats, the heart rate remained a steady value and no significant differences were found along the baseline-stroke-recovery process (section 4.24). A stable heart rate may indicate a reliable cardio output during the measurement and ensure that arterial BFV

changes are caused by stroke surgery only. Similar as the heart rate, the body weights of the two group rats did not change significantly after the stroke surgery, and kept relatively stable throughout the experiment period (Fig 4.6). Body weight is generally regarded as an important index to evaluate the general physiological condition and the degree of recovery of experimental rats after stroke (Dittmar et al. 2003). Its development was correlated to histopathologically changes observed brain damage (Palmer et al. 2001). The insignificant changes of body weight in both group rats may be an indication of fast recovery after stroke insult.

After stroke, both group rats showed motor deficits, which level was evaluated with the De Ryck's behavioral test. Although the inter-group significant differences on behavioral scores compared with the first day (D1) after stroke were shown from D2 and D3 in ischemic stroke rats and ICH stroke rats, respectively, no significant difference was found between the two groups (Fig 4.7). This may indicate that both types of stroke models produce comparable motor deficits with similar recovery process.

The brain infarct volume measured with TTC staining technique showed great difference between two groups: 148 ± 56 mm^3 in the ischemic group and 6.8 ± 0.7 mm^3 in the ICH group (Fig 4.8). Although considerable hematoma was observed in brain slicing (Fig 4.8b), comparable infarct volume was not revealed. This is due to the low sensitivity of TTC in infarct volume detection in ICH model (Patel et al. 1999). This limitation was due to the original purpose of

current study to compare the ischemic stroke and hemorrhagic stroke models with the same evaluation methods, and should be implemented in future studies.

5.1.6 Summary

The current study compared the ischemic stroke model and the hemorrhagic stroke model with the application of ultrasonography technique and the results is summarized in Table 5.1. The comparison results may indicate that ischemic strokes, even the occlusion only occurs in a single artery, can lead to significant changes of BFV in other cerebral arteries, while ICH stroke mainly affect the arteries supplying the hemorrhagic tissues. It was also suggested that collagenase used in ICH model produced an exaggerated inflammatory response and was directly toxic to neurons (Xi et al. 2006). Concerning the stroke incidences of ischemic strokes (> 80%) and hemorrhagic strokes (< 20%) and the toxicity induced by the collagenase in the hemorrhagic stroke model, the ischemic stroke model was selected in the following experiment.

Table 5.1 Comparisons of BFV changes, motor recovery, behavioral score, body weigh and TTC staining results between ischemia stroke and hemorrhage stroke

	BFV changes	Significant Motor recovery	Behavioral score 24 hrs after stroke (D1)	Body weight	TTC staining
ICH	Significant differences were found in RMCA (D0-D1) and RPCA (D0), and BFV in all vessles return to baseline values from D2	From D3	6 ± 1.5 (significantly decreased from baseline: 62.5% decrease)	Not significantly changed	Infarct volume revealed: 6.8 ± 0.7 mm^3
Ischemia	Reversed flow was found in RICA; significant differences were found in RMCA (D0-D4), LMCA (D0), LICA (D1-D4), BA (D1-D4)	From D2	5.25 ± 1.71 (significantly decreased from baseline: 67.2% decrease)	Not significantly changed	Infarct volume revealed: 148 ± 56 mm^3

ICA: internal carotid artery; MCA: middle cerebral artery; PCA: right posterior cerebral artery; BA: basilar artery.

D0 represents the day of ischemia/0.5 hr after ICH surgery, and D1 represents one day after the surgery, and the following days.

5.2 Comparison of the Effects of Different Exercise Interventions as Stroke Rehabilitation Tools in a Rat Cerebral Ischemia Model

This study was intended to compare the effectiveness between voluntary, involuntary, and forced exercises under similar training intensity with the evaluation of the motor recovery, and their functions in regulating brain BDNF and serum corticosterone secretion after brain ischemia in a rat model. Our results showed that all three types of exercise applied in this study had better motor recovery than the control group. The voluntary exercise induced higher

hippocampal BDNF level, better improvement in motor recovery when compared with involuntary exercise, forced exercise, and control groups. Moreover, our results also demonstrated that forced exercise could induce stress, which probably down-regulated BDNF levels in the brain.

5.2.1 Body Weight

After the MCAo/r surgery, all groups (V-Ex, I-Ex, F-Ex, and Con) rats had a significantly decreased body weight from average 300g to average 220g. The acute drop of body weight may be due to three reasons: (1) the surgery caused the neurological impairments and disturbed the whole body metabolism; (2) the surgery led to pain and impairment of mastication and swallowing in rats, consequently resulting in reduced food and water consumption (Dittmar et al. 2003); and (3) neuron damage caused anorexia and reduction of nutrition intake due to functional disability of upper arm (Choe et al. 2004). The body weight of all group rats gradually increased after the surgery along the experimental period, suggesting that the neurological impairments and motor deficits recovered gradually, spontaneously and/or due to exercise interventions. In addition, no significant difference was found among groups, indicating that the current exercise regimens did not exaggerate the stroke complications (Matsuda et al. 2011).

5.2.2 Brain-Derived Neurotrophic Factor (BDNF) Regulation

BDNF is a powerful differentiation factor distributed widely in the central nervous system (CNS), and it is generally recognized as critical moderators in the mechanism of how physical activity impacts neural function at the cellular and molecular levels (Gomez-Pinilla et al. 2001). Physical activity can induce the expression of neurotrophic factors in the hippocampus and other brain regions, and BDNF is most abundant in the hippocampus (Gomez-Pinilla et al. 1998; Binder and Scharfman 2004). In accordance with this statement, our results also showed that BDNF concentrations were higher in the hippocampus than in the cortex and striatum in all four groups (Fig 4.12b). BDNF is involved in various aspects of neuroplasticity, such as neurogenesis, long-term potentiation (LTP), which is a cellular model of learning, memory, and even mood changes (Shoval and Weizman 2005; Yamada et al. 2002). LTP formation has been suggested as BDNF-dependent, since experiments have demonstrated that LTP is impaired in BDNF-deficient mice, but can be restored with BDNF infusion (Noble et al. 1999). BDNF also shows characteristics of neuroprotection in that it promotes survival of hippocampal, striatal, and septal neurons in culture and *in vivo* by protecting the brain against insults such as ischemia and axotomy (Noble et al. 1999; Leung et al. 2007). Studies showed that the 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). Recent studies suggested that exercise-induced enhancement in learning and memory was

dependent on an increased hippocampal BDNF level, and the behavioral recovery was also correlated with a cell proliferation in the rat hippocampus (Vaynman and Gomez-Pinilla 2005; Shoval and Weizman 2005). Among different exercise paradigms, the voluntary wheel running, the involuntary functional electrical stimulation, and the forced treadmill running have been individually demonstrated to correlate the up-regulated BNDF level and motor function recovery (Adlard et al. 2004; Burnett et al. 2006; Huang et al. 2006). On the other hand, physical exercise can also induce down-regulation of BNDF through the effects of stress. Accrued evidence has demonstrated that stress, which can be induced by high-intensity exercise, or corticosterone administration reduces BDNF mRNA expression in hippocampus (Smith et al. 1995; Schaaf et al. 1997). In an animal study, treadmill running at a speed of 25m/min was shown to elevate stress level and depress hippocampal BDNF protein expression (Soya et al. 2007). Due to the inhibitory effect of stress in BDNF regulation, experiments using exercise training as a rehabilitation intervention try to minimize the stress caused by the exercise. Decreased or adjustable training intensity and pre-familiarization were suggested as effective tools (Soya et al. 2007; Matsuda et al. 2011; Huang et al. 2006). Consistent with these studies, a moderate exercise intensity with accommodation to eliminate the stress effect in different exercise groups was applied in the present study, and the results are discussed in later sections.

The role of BDNF in regulation of neuronal survival, angiogenesis, and synaptic function has been well recognized (Aguado et al. 2003; Gorski et al. 2003). The

way BDNF facilitates motor recovery after brain ischemia has been actively investigated. After injury, rehabilitation and motor learning aid in reorganizing cortical maps, which is thought to be one of the main effectors of recovery after stroke (Monfils et al. 2005). It is generally believed that BDNF contributes greatly in the cortical map reorganization, synaptogenesis increase, dendritic spine formation and branching enhancement, and other forms of neuronal plasticity implicated in recovery after stroke (Ploughman et al. 2009). Considering the crucial role of BDNF in mediating motor learning, we evaluated and discussed the motor function recovery with primary inspection of brain BDNF levels in different exercise-intervention groups.

5.2.3 Spontaneous Recovery

Neurological deficits induced by focal cerebral ischemia are generally characterized by sensorimotor dysfunction, which have been clearly documented by previous literatures (Bederson et al. 1986; Markgraf et al. 1992; Belayev et al. 1996). In the current study, De Ryck's behavioral test scores and beam walking test scores gradually increased along the experimental period in both exercise-intervened groups and control group, indicating motor recovery (Fig 4.10, Fig 4.11). The improvement of performance in behavioral test and beam walking test in the Con group rats should be primarily due to spontaneous recovery following ischemic injury of the brain, which was supported by previous studies (Yang et al. 2003b; Bland et al. 2001; Belayev et al. 1996). With

spontaneous recovery, reduced infarction of the ischemic brain, increased cell proliferation and neurogenesis, and increased excitability in bilateral cortical hemispheres can be achieved. In Yang et al.'s study, the infarct volume was significantly larger in the rats sacrificed 24 hours after MCAo/r than in those sacrificed 2 weeks later without any intervention (Yang et al. 2003b). The spontaneous recovery was also shown as 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). In a clinical study, a greater neuronal activity in both ipsilateral and contralateral cortexes was found in the early stage after stroke, also suggesting that the spontaneous recovery occurred (Schaechter 2004).

5.2.4 Effects of Voluntary Wheel Running

Wheel running is generally regarded as a type of voluntary exercise in animal models, and it does not activate systemic stress (Yanagita et al. 2007). The beneficial effects of voluntary wheel running were demonstrated in blunting stress response and strengthening adrenal function in healthy rats (Dishman et al. 1998; Campbell et al. 2009). Some study suggests that the voluntary wheel running can attenuate the stress response in cardiovascular disorders rats while the treadmill running not (Morimoto et al. 2000). In accordance with this, our result also showed that the V-Ex group rats had significantly lower corticosterone/stress level than the F-Ex and I-Ex group rats did, and had no

significant difference compared with the Con group rats (Fig. 4.13). Voluntary wheel running suppresses the stress response by increasing the adrenal sensitivity to adrenocorticotropic hormone (ACTH), which is released by pituitary gland when the body's stress response system is activated (Droste et al. 2003). Such enhanced adrenal sensitivity can prevent the stress response system from exercise-induced hypersensitivity and consequently inhibit the downregulation of neurotrophic factors in the hippocampus (Duclos et al. 1998; Fediuc et al. 2006). Through this indirect pathway, voluntary wheel running increases the BNDF availability in the hippocampus. On the other hand, voluntary exercise can also directly encourage hippocampal BDNF secretion. Evidence from other studies has revealed the effectiveness of voluntary wheel running in the up-regulation of BDNF in the rat hippocampus (Neeper et al. 1995; Berchtold et al. 2001; Marin et al. 2003; Adlard et al. 2004). Similar results have been confirmed in a study comparing various rat strains including Brown Norway, Sprague-Dawley, Dark Aguouti, and Piebald-Viral-Glaxo, indicating that BDNF levels increase with voluntary exercise regardless the strain differences in voluntary activity (Johnson and Mitchell 2003). Alternatively, inhibiting BDNF action can actually block the beneficial effects of running wheel exercise on the performance in the Morris water maze task (Vaynman et al. 2004). In the current study, the hippocampal BDNF level in the V-Ex group rats was significantly higher than those in the Con and F-Ex groups after the 7-day exercise interventions after brain ischemia (Fig 4.12a), which result has not been reported in previous literatures. Although the

underlying mechanisms of voluntary exercise induced upregulation of hippocampal BNDF are still largely unexplored, several possible explanations are listed in the following. First, voluntary exercise may stimulate hippocampal neural activity that increases BDNF gene expression. As first reported by Vanderwolf in 1969, voluntary exercise activates a persistent firing pattern (known as theta-rhythm) in the rat hippocampus, and this firing pattern is dependent on cholinergic and GABAergic neurons (Vanderwolf 1696). Such theta bursts lead to the secretion of BDNF in the hippocampus (Carro et al. 2000). Second, exercise evokes various molecules that have a recognized interaction with BDNF and are important for synaptic plasticity. For example, it has been suggested that an elevation of norepinephrine during voluntary exercise may increase its coupled receptor, which in turn activates downstream intracellular signaling pathways. This norepinephrine-receptor binding further activates the phosphorylated protein-kinase-signaling pathway that functions on the promoter region of the BDNF gene and increases the BDNF mRNA and protein levels (Jovanovic et al. 1996; Chen and Russo-Neustadt 2005). Third, an elevated free calcium ion level in the brain during exercise may also upregulate BDNF gene expression via the activation of cyclic adenosine monophosphate response element-binding protein (Shen et al. 2001; West et al. 2001). Together, these evidences may explain our results that the V-Ex group rats had the highest hippocampal BDNF level and the highest last-day scores in both the behavioral and the beam walking tests (Fig 4.10, Fig 4.11, Fig 4.12a). Parallel to this result,

voluntary wheel running was demonstrated to speed the motor function recovery after brain ischemia (Zhao et al. 2005). In addition, our results also echoed previous publications that the motor recovery was more related to the hippocampal BDNF level than the striatal and cortical BDNF levels, as the V-Ex group rats had high hippocampal but low striatal and cortical BDNF levels.

Interestingly, it was shown that the amount of exercise was significantly and positively correlated with hippocampal BDNF expression (Neeper et al. 1995). In the current experiment, the quantity of exercise was fixed in the F-Ex group (30 min treadmill running for a total of 600m) and the I-Ex group (30 min electrical stimulation with similar muscle contraction pattern as treadmill rats running at a speed of 20 m/min), whereas the running distance of the V-Ex group was flexible and dependent on rats who were free to run. The V-Ex group rats had similar running distance as that of the F-Ex group rats on I1 (1^{st} day after MCAo/r surgery), but gradually increased their running distance in the remaining experimental period (Fig 3.13b). This result may be another reason of better functional recovery in the V-Ex group: excess running exercise activated additional hippocampal BDNF release, which in turn hastened neurogenesis and motor recovery. The favorable effects from extra running quantity, however, may only exist in voluntary exercise, when in treadmill exercise such abundant exercise amount was shown to accompany with elevated stress level and reduced BDNF concentration (Huang et al. 2006; Soya et al. 2007).

5.2.5 Effects of Involuntary Functional Electrical Stimulation

The functional electrical stimulation (FES) was recently applied in animal models to study its effect of stroke rehabilitation, and the literatures regarding the physiological changes in the brain induced by FES are limited. It is generally believed that FES affects central nervous system by modifying the excitability of specific motor neurons (Chae and Yu 1999). In this study, the results showed that the I-Ex group rats had the highest striatal and cortical BDNF levels, and also a significantly higher hippocampal BDNF level than F-Ex and Con group rats (Fig 4.12a). This may be attributed to the fact that electrical stimulation of the affected muscle groups brings about a series of physiological effects, including an increase in metabolism and cerebral blood flow (CBF) (Park et al. 2004). A study using global cerebral ischemia model with rats showed that the electrical stimulation on median nerve was associated with decreased apoptotic neuronal injury in the hippocampus, and it was proposed 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 (Buitrago et al. 2004). Electrical stimulation of peripheral nerves induces contraction of innervated skeletal muscles via motor nerve fibers, which consequently activates the CNS via sensory nerve fibers. With the stimulation of CNS, an increase of CBF is expected, and such linkage between neuron activities and CBF is also called activation-flow coupling. Burnett et al. demonstrated that, with the electrical stimulation of the rat's forepaw, the activation-flow coupling response was preserved over a broad range

of baseline flow values during the MCAo/r (Burnett et al. 2006). As a result, a reasonable explanation is that the electrical stimulation increases CBF, which consequently activates neurons and up-regulates chemicals such as trophic factors in these neurons.

It was also noted that the I-Ex group rats had better performance in the behavioral test and beam walking test when compared with the F-Ex and Con group rats (Fig 4.10, Fig 4.11). Such superior motor function recovery may be caused by the functional reorganization and neuromodulation of the brain region. Functional magnetic resonance imaging technique revealed the activation at unaffected contralateral sensorimotor cortex as well as the regions lateral to the lesion site during electrical stimulation was administered on the paretic hindlimb (Abo et al. 2001). Another possible explanation is that the FES stimulates the BDNF secretion in the innervated muscle fibers, and the local enhanced neuroplasticity promotes muscle function recovery. Park et al. illustrated that electrical stimulation of the sciatic nerve elevated BDNF level in the soleus and medial gastrocnemius muscles (Park et al. 2004). This increased BNDF level was shown to promote the development of electrophysiological properties of the neuromuscular synapse, and may enhance the potential of innervation of motoneurons in the impaired limb (Wang et al. 1995; Gomez-Pinilla et al. 2001).

5.2.6 Effects of Forced Treadmill Exercise

The treadmill exercise requires a repetitive locomotor performance similar to voluntary wheel exercise and represents a functional activity which is purposeful bilateral hemisphere activity. Clinical studies demonstrated that treadmill training was effective with regard to restoration of gait ability and improvement of gross motor efficiency (Harris et al. 2001). The effects of treadmill exercise in encouraging motor recovery compared with spontaneous recovery have been substantiated in various studies (Wang et al. 2001; Zhang et al. 2010; Matsuda et al. 2011; Yang et al. 2003b). A treadmill running at a speed of 20 m/min for 30 minutes per day for as least two weeks significantly reduced the infarction volume and edema caused by MCAo when performed before stroke (Wang et al. 2001). Similarly, pre-ischemia treadmill exercise with the same intensity as in Wang et al.'s study for two weeks can also significantly reduce the neurological deficits (Zhang et al. 2010). The treadmill training performed after the stroke has also presented beneficial effects (Matsuda et al. 2011; Yang et al. 2003b). Matsuda et al. showed that rats with treadmill running at a speed of 3-18 m/min for 20 minutes per day for maximum 28 days had significantly reduced infarct volume and neurological deficits (Matsuda et al. 2011). Moreover, rats ran on a treadmill at a speed of 20 m/min for 30 minutes per day also showed a smaller brain infarct and neurological deficits with only 5 days of training (Yang et al. 2003b). These evidences have supported our result that the rats with treadmill exercise had better performance in the behavioral test and beam walking test than

the control group (Fig 4.10, Fig 4.11). Although the underlying mechanism has not been fully investigated, researches generally believe that neuronal growth factors and angiogenesis play roles in the neuroprotective effects of physical exercise against the damage caused by cerebral ischemia (Matsuda et al. 2011). Similar as other physical activities, treadmill exercise induces an increased blood vessel density and neurogenesis in brain, thus promoting the synaptic function recovery (Griesbach et al. 2004). The running intensity was noted, as it was indicated that the intensity of training appears to be one of the important factors that contribute to early exclusive use-dependent exaggeration of injury (Bland et al. 2000). In addition, it was suggested that the beneficial effects of treadmill exercise only existed in low-intensity treadmill (15 m/min) running rats, and moderate-intensity treadmill (25 m/min) would elevate serum corticosterone and evoke stress responses (Soya et al. 2007). In our results, although the intensity of treadmill running was set at a low-moderate level (20 m/min), the stress response was still significant, which presented as the particularly high serum corticosterone level in the F-Ex group rats (Fig 4.13). It was explained by previous study that during forced exercise such as treadmill, animals experience a loss of control over their activity pattern, thereby causing severe psychological stress and major disruption in the normal stress response system (Fediuc et al. 2006). More specifically, forced exercise studies have shown an enlargement of the adrenal glands, elevations in diurnal corticosterone levels, and a reduction in the circulating ACTH, suggesting a reduced adrenal sensitivity to ACTH

(White-Welkley et al. 1996; Fediuc et al. 2006). The role of adrenal sensitivity to ACTH in brain BDNF regulation was discussed in previous section 5.2.4. Previous studies have suggested that stress during treadmill training counteracted the beneficial effects of exercise on the up-regulation of BDNF (Cechetti et al. 2008; Huang et al. 2006). Considering the significantly high serum corticosterone level and extremely low hippocampal BDNF levels in the F-Ex group rats, we speculate that corticosterone released during the forced exercise has downregulated the brain BDNF expression, which in turn attenuates the effects of exercise in facilitating functional recovery after brain ischemia.

CHAPTER 6

CONCLUSIONS

This study firstly investigate the ischemic stroke rat model and the hemorrhagic stroke rat model primarily regarding the differences of cerebral blood flow changes using ultrasonography technique, and found that the whole cerebral vascular circulation is disturbed in the ischemic stroke model, whereas in the hemorrhagic stroke model only arteries supplying the hemorrhagic tissues are affected. Afterwards, the ischemic stroke rat model was applied to compare the rehabilitation effects of voluntary wheel exercise, involuntary functional electrical stimulation exercise, and forced treadmill exercise. The results showed that the V-Ex group had significantly higher score in the behavioral test than all the other groups and significantly higher hippocampal BDNF concentration than the F-Ex and Con groups. On the other hand, the F-Ex group had significantly higher serum corticosterone level than the other groups. These results suggested that the voluntary exercise was the most effective intervention in upregulating the hippocampal BDNF level, and facilitating motor recovery. Rats that exercised voluntarily also showed less corticosterone stress response than the other groups. The results also suggested that the forced exercise group was the least preferred intervention with high stress, low brain BDNF levels and less motor recovery.

Several areas for future research could be suggested based on the findings of this study.

Firstly, in this study, fixed exercise intensity was administered to the involuntary exercise group and the forced exercise group. It would be interesting to examine the effects of different exercise intensities such as duration of running and frequency of electric stimulation.

Secondly, as the effects of voluntary, involuntary and forced exercises on motor function recovery have been investigated in this study, a combination of different exercise schemes may be conducted on stroke rats to explore whether there will be a synergistic effect. It might be possible that voluntary exercised rats with functional electrical stimulation will obtain even better motor function recovery than voluntary exercised rats after stroke.

Thirdly, since the voluntary exercise was the most effective one to facilitate motor recovery and up-regulate hippocampal BDNF level, future study may explore the relationship between the amount of exercise and recovery level in the voluntarily exercised rats.

Finally, the underlying mechanism of the effects of BDNF on motor function recovery is still largely unexplored, and investigation of relationship between BDNF and other neurochemicals may bring about valuable clinical implications in stroke rehabilitation.

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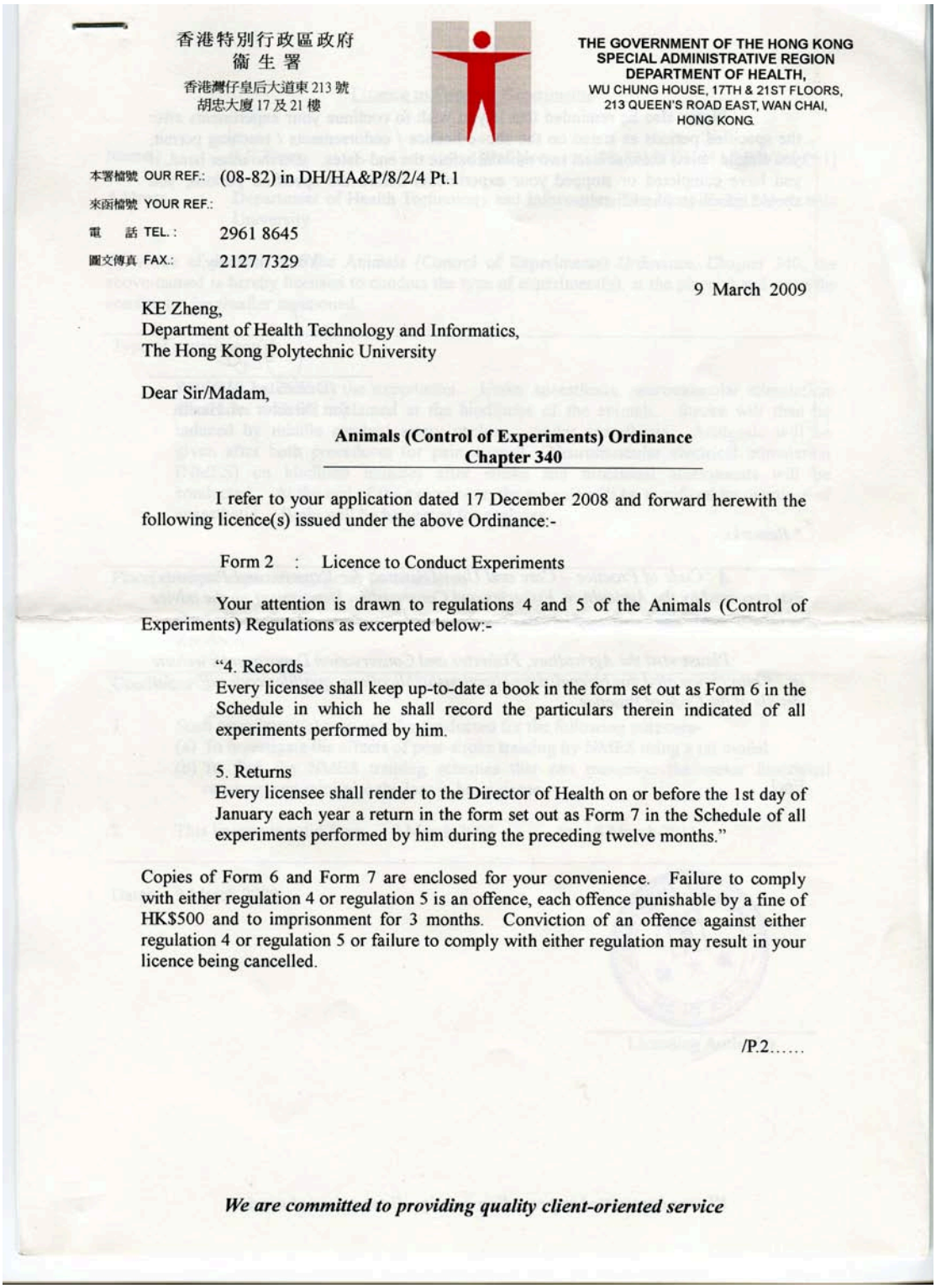
Appendix

1. Ethic Approvals



香港特別行政區政府
衞生署
香港灣仔皇后大道東213號
胡忠大廈17及21樓

THE GOVERNMENT OF THE HONG KONG SPECIAL ADMINISTRATIVE REGION
DEPARTMENT OF HEALTH,
WU CHUNG HOUSE, 17TH & 21ST FLOORS,
213 QUEEN'S ROAD EAST, WAN CHAI,
HONG KONG.

本署檔號 OUR REF.: (08-82) in DH/HA&P/8/2/4 Pt.1
來函檔號 YOUR REF.:
電　話 TEL.: 2961 8645
圖文傳真 FAX.: 2127 7329

9 March 2009

KE Zheng,
Department of Health Technology and Informatics,
The Hong Kong Polytechnic University

Dear Sir/Madam,

Animals (Control of Experiments) Ordinance
Chapter 340

I refer to your application dated 17 December 2008 and forward herewith the following licence(s) issued under the above Ordinance:-

Form 2 : Licence to Conduct Experiments

Your attention is drawn to regulations 4 and 5 of the Animals (Control of Experiments) Regulations as excerpted below:-

"4. Records
Every licensee shall keep up-to-date a book in the form set out as Form 6 in the Schedule in which he shall record the particulars therein indicated of all experiments performed by him.

5. Returns
Every licensee shall render to the Director of Health on or before the 1st day of January each year a return in the form set out as Form 7 in the Schedule of all experiments performed by him during the preceding twelve months."

Copies of Form 6 and Form 7 are enclosed for your convenience. Failure to comply with either regulation 4 or regulation 5 is an offence, each offence punishable by a fine of HK$500 and to imprisonment for 3 months. Conviction of an offence against either regulation 4 or regulation 5 or failure to comply with either regulation may result in your licence being cancelled.

/P.2.....

We are committed to providing quality client-oriented service

Please also be reminded that if you wish to continue your experiments after the specified periods as stated on the above licence / endorsements / teaching permit, you should renew them at least two months before the end-dates. On the other hand, if you have completed or stopped your experiments before the specified periods, you should inform us immediately.

Yours sincerely,

(Dr Emily LEUNG)
for Director of Health

** Remarks:-*

A "Code of Practice – Care and Use of Animals for Experimental Purposes" was prepared by the Agriculture, Fisheries and Conservation Department on the advice of the Animal Welfare Advisory Group.

Please visit the Agriculture, Fisheries and Conservation Department's website at <http://www.afcd.gov.hk/english/publications/publications_qua/files/code.pdf> for details of the Code of Practice.

Encl.

We are committed to providing quality client-oriented service

Form 2

Licence to Conduct Experiments

Name : KE Zheng [Ref No.: (08-82) in DH/HA&P/8/2/4 Pt.1]

Address : Department of Health Technology and Informatics, The Hong Kong Polytechnic University

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)

Rats will be used in the experiment. Under anaesthesia, neuromuscular stimulation electrodes will be implanted at the hindlimbs of the animals. Stroke will then be induced by middle cerebral artery occlusion under anaesthesia. Analgesic will be given after both procedures for pain control. Neuromuscular electrical stimulation (NMES) on hindlimb muscles after stroke and functional assessments will be conducted. At the end of the experiment, the animals will be sacrificed by overdose of anaesthetic. Brain will be harvested for analyses.

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

Centralised Animal Facilities, The Hong Kong Polytechnic University, Hung Hom, Kowloon

Conditions

1. Such experiment(s) may only be conducted for the following purposes-
   (a) To investigate the effects of post-stroke training by NMES using a rat model.
   (b) To find the NMES training schemes that can maximise the motor functional recovery and minimise the loss in brain tissue.

2. This licence is valid from 9 March 2009 to 8 March 2011

Dated 9 March 2009

DEPARTMENT OF HEALTH 衛生署 HONG KONG

Licensing Authority

2. Procedure of brain tissue treatment and ELISA test (procedures followed the instruction from the manufacturer, and figures were calculated according to the experimental setup)

Sample Preparation

1. Harvest of cerebral cortex, striatum and hippocampus, immersed in 1 ml lysis buffer.
2. Homogenization.
3. Dilute 200 µl homogenate in 800 µl DPBS.
4. Acidify homogenate to ~3.0 using 1N HCL. (1µl 1N HCl per 50 µl sample)
5. Incubate at room temperature for 15 min.
6. Neutralize with 1N NaOH to pH ~7.6. (1µl 1N NaOH for 50 µl sample)
7. Microfuge at 14000 rpm for 3 min.
8. Collect the supernatant and stored at -20 ^{o}C.

ELISA

1. 10 µl of the Anti-BDNF mAb to 9.99 ml of carbonate coating buffer. Add 100 µl to each well. NOTE: Keep the undiluted Anti-BDNF mAB on ice.
2. Seal the wells and incubate without shaking overnight at 4 ^{o}C.
3. 8.8 ml of Block & Sample 5X Buffer to 35.2 ml of DI water (Aseptic transfer technique).
4. Flick out the contents of the wells and wash with TBST wash buffer.
5. Add 200 µl of Block & Sample 1X Buffer to each well.
6. Incubate at room temperature for 1 hr without shaking.
7. Thaw sample in 4 ^{o}C refrigerator.
8. 2 µl undiluted BDNF Standard to 78 µl Block & Sample 1X Buffer. (Solution A)
    1) 10 µl : 490 µl Block & Sample 1X Buffer (500 pg/ml)
    2) 10 µl : 615 µl Block & Sample 1X Buffer (400 pg/ml)
    3) 10 µl : 823 µl Block & Sample 1X Buffer (300 pg/ml)
    4) 10 µl : 990 µl Block & Sample 1X Buffer (250 pg/ml)
    5) 5 µl : 620 µl Block & Sample 1X Buffer (200 pg/ml)
    6) 5 µl : 1245 µl Block & Sample 1X Buffer (100 pg/ml)
    7) 50 µl Solution 1) : 450 µl Block & Sample 1X Buffer (50 pg/ml)

    NOTE: Keep the undiluted BDNF Standard on ice.
9. Flick out the contents of the wells. Wash once with TBST wash buffer.
10. Designate two columns of wells for the standard cure. (100 µl/well)

	11	12
A	500 pg/ml	500 pg/ml
B	400 pg/ml	400 pg/ml
C	300 pg/ml	300 pg/ml
D	250 pg/ml	250 pg/ml
E	200 pg/ml	200 pg/ml

F	100 pg/ml	100 pg/ml
G	50 pg/ml	50 pg/ml
H	1X B&S Buffer	1X B&S Buffer

11. Add 100 µl of the samples to wells. Seal the wells with a plate sealer and incubate the plate for two hours at room temperature with shaking (400 ± 100 rpm).
12. 16 µl Anti-Human BDNF pAb to 7.984 ml of Block & Sample 1X Buffer.
13. Wash the plate 5 times with TBST wash buffer.
14. Add 100 µl of the diluted Anti-Human BDNF pAb to each well. NOTE: Keep the undiluted Anti-Human BDNF pAb on ice.
15. Seal the wells with a plate sealer and incubate for 2 hrs at room temperature with shaking.
16. Wash the plate 5 times with TBST wash buffer.
17. 41 µl Anti-IgY HRP Conjugate to 8.159 ml of Block & Sample 1X Buffer.
18. Add 100 µl of the diluted Anti-IgY HRP Conjugate to each well. NOTE: Keep the undiluted Anti-IgY HRP on ice
19. Incubate for 1 hr at room temperature with shaking (400 ± 100 rpm).
20. Equilibrate the TMB One Solution to room temperature.
21. Wash the plate five times with TBST wash buffer.
22. Add 100 µl of the room temperature TMB One Solution to each well.
23. Incubate at room temperature with shaking for 10 min. (Blue)
24. Add 100 µl of 1N HCl to the wells in the same order in which substrate was added in the previous step. (Yellow)
25. Record the absorbance at 450 nm on a plate reader within 30 min of stopping the reaction. NOTE: The exterior bottom of the plate must be optically clean for accurate measurement. Wipe the exterior bottom with 70% ethanol if necessary.