Changes in the Central Nervous System after Bilateral Occlusion of the Common Carotid Arteries in the Hypertensive Rats and the Effect of Pien Tze Huang

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Abstract of thesis entitled:

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Brain stroke is considered as one of the three diseases that threaten human health all over the world. Hypertension and cerebral arteriosclerosis are thought to be the most dangerous risk factors of brain stroke, and they frequently occur together, leading to ischemia of brain tissue. Unfortunately, it is not clear whether the pathological changes resulting from hypertension are related to those resulting from cerebral arteriosclerosis. There have been no ideal animal models mimicking the pathological changes in such a combined condition. In this thesis, an animal model of hypertension combined with cerebral arteriosclerosis in rats was established by occlusion of both the left and right common carotid arteries in spontaneous hypertension rats. Pien Tze Huang (PTH), a reputed traditional Chinese medicinal complex, contains Radix notoginseng, snake bile, calculus bovis, and musk and some other components that are known to protect vessels and cells from injuries. Since different tissue injuries share many common cellular mechanisms, the protection by PTH to in nerves and the circulation systems may also be benefical to cerebrovascular conditions as well. In present experiments, PTH was used to treat hypertension rats that also developed chronic brain ischemia as a result of the bilateral carotid occlusion, and its protective role for neurons and blood vessels was investiaged.

In the initial part of the work, patients from clinics in two cities in South and North China were compared and analysed; they had been suffering from brain ischemic stroke. About two thirds of the stroke patients were found to have hypertension before the onset of stroke. Their prognosis was significantly worse than those stroke patients without hypertension. In the hypertensive rats with occluded arteries, mean of functional magnetic resonance imaging (fMRI) examination showed that brain blood flow was very weak or even transiently became undetectable at the beginning of the acute stage of brain ischemia, but was restored one hour after the occlusion surgery. In addition, pathological changes in brains of hypertensive rats with induced brain ischemia (carotid occlusion) were examined by Nissl staining, TUNEL staining, cell death ELISA and anti-oxidation enzymes. At day 15 after ischemia, a large number of pyramid cells in the hippocampus of SHR were lost and a great deal of apoptotic cells were found in the CA1 of the hippocampus, while activities of some enzyme including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) were increased. At day 30 and 60, some degenerative changes appeared to have subsided and the cells appeared morphologically normal. The activities of the above enzymes were also decreased at day 60. In WKY control rats with normal blood pressure, neurons in the CA1 were found less damaged after the bilateral carotid occlusion. It was found that apoptotic and dead cells were significantly reduced in rats with hypertension combined with chronic brain ischemia if they had been pre-treated with PTH. Moreover, brain stroke damage was less severe in this pretreated rats.

From the data above, more severe damage could be caused by hypertension combined with chronic ischemia. The model of SHR with bilaterally occluded common carotid artery can be used to study pathological changes resulted from hypertension combined with chronic ischemia. PTH was able to protect neurons in stroke.

中文摘要

腦卒中是當今世界危害人類健康的三大疾病之一，高血壓和腦動脈硬化是腦卒中最重要的危險因素，兩者常常合併存在，造成腦組織慢性缺血。然而，目前對於高血壓合併腦動脈硬化所造成的腦組織的病理改變還不很清楚，也沒有能夠模仿兩種病理狀態合併存在的理想的動物模型。本實驗擬通過阻斷自發性高血壓大鼠（SHR）的雙側頸總動脈造成慢性腦缺血合併高血壓的模型，並觀察其腦組織的病理改變。片仔癀是一種名貴的複方中成藥，目前，有關片仔癀對神經和血管保護作用，在臨床和基礎研究中均未見報道。然而，大量的研究證明片仔癀的主要組成成分三七，蛇膽，牛黃和麝香均有明顯的神經和血管保護作用，這就提示片仔癀也可能具有神經和血管保護作用。本實驗擬用片仔癀預防性治療高血壓合併慢性腦缺血，初步觀察片仔癀的神經血管保護作用。

首先我們對中國大陸華北和華南兩城市的缺血性腦卒中患者做了臨床分析，果顯示 68.28%的患者發病前合併有高血壓，而且比沒有高血壓的患者預後差。我們又應用功能性磁共振技術觀察了 SHR 在缺血急性期的腦血流改變，發現經過短暫的腦血流信號消失後，在缺血後 1 小時又恢復血流信號。接著，我們應用 Nissl 染色、TUNEL 染色、ELISA 和生化的方法檢測了高血壓合併慢性腦缺血大鼠腦部的病理改變，發現在缺血後 15 天，海馬 CA1 區錐體細胞大量丟失，出現大量凋亡細胞，超氧化物歧化酶、過氧化氫酶、谷胱甘肽過氧化物酶表現出較高的活性，在缺血後 30 天和 60 天，組織結構基本恢復正常，酶的活性在缺血後 60 天有不同程度的下降。在血壓正常的 Wistar-Kyoto 大鼠，海馬 CA1 區則表現出逐漸受損的趨勢。在應用片仔癀對慢性腦缺血合

併高血壓和腦卒中進行預防性治療後，發現大鼠海馬的凋亡和死亡明顯降低，而且可以延緩腦卒中的發生和降低腦卒中的嚴重程度。

由以上的結果可見，慢性腦缺血合併高血壓會對缺血腦組織造成更大的損害， 阻斷 SHR 雙側頸總動脈可以作為研究慢性腦缺血合併高血壓病理狀態的動物模型； 片仔癀具有明顯的神經保護作用。

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Zhang Lihong

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List of abbreviations

2-vessel occlusion; 2VO	Bilateral common carotid artery occlusion
ABTS	2,2'-azino-di-[3-ethylbenzthiazoline sulfonate
AEEC	Animal Experimentation Ethics Committee
Bax	Bcl-2 associated X protein
Bcl-2	B-cell leukemia/lymphoma-2
BOLD	Blood-oxygenation-level-dependent
cAMP	Monophosphate
CAT	Catalase
CBF	Cerebral blood flow
CCA	Common carotid arteries
ChAT	Acetylcholine transferase
cm	Centimeter
CT	Computed tomography
CVD	Cerebrovascular disease
d	Day
DAB	3,3′-Diaminobenzidine tetrahydrochloride hydrate
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EEAs	Excitatory amino acids
ELISA	Enzyme-linked immunosorbant assay
ET	Endothelin
fMRI	Functional magnetic resonance imaging
g	Gram
GFAP	Glial fibrillary acidic protein
Glu	Glucose
GPx	Glutathione peroxidase
GSH	Glutathione
h	Hour
IDH	Diastolic hypertension

ISH	Systolic hypertension
Lac	Lactate
LASC	Laboratory Animal Services Centre
MDA	Malondialdehyde
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimole
MONICA	Multinational Monitoring of trends and determinants in cardiovascular disease
MRI	Magnetic resonance imaging
NMDA	N-methyl-D-aspartic acid
OD	Optical density
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PNS	Panax notoginseng saponins
POD	Peroxidase
PTH	Pien Tze Huang
PTS	Panaxatriol saponin
RHRSP	Stroke-prone renovascular hypertensive rat
s	second
SD	Standard deviation
SD rat	Sprague-Dawley rat
SDS	Sodium dodecyl sulphate
SHR	Spontaneously hypertensive rat
SHRsp	Stroke-prone spontaneously hypertensive rat
SOD	Superoxide dismutase
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	taurodeoxycholic acid
TdT	Terminal deoxynucleotidyl transferase
TMB	3, 3', 5, 5'tetramethyl-benzidine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

TXA_2	Thromboxane A_2
VSMC	Vascular smooth muscle cell
WHO	World Health Organization
WKY rat	Wistar-Kyoto rat

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Chapter 1 General introduction

1.1 The epidemiological study of ischemic stroke in China

Stroke is a severe and acute cerebrovascular disease (CVD), which has a high incidence and high mortality (Thorvaldsen, et al., 1995; Feigin, 2005) and there is a limited time frame of treatment (Furlan, et al., 1999; Durai, et al., 2007). It is one of the three lethal diseases in the world. The expensive cost in therapy of stroke has brought heavy burden on families and societies (Durai, et al., 2007; Wang, et al., 2007). Based on the characteristics of pathology, strokes can be classified into ischemic stroke and hemorrhagic stroke. The ischemic stroke is caused by loss of blood supply, and lack of blood and oxygen leads to local brain tissue cell death. The incidence of ischemic stroke is around 75%-80% of all stroke in the western countries (Akopov and Cohen, 2003; Ovbiagele, et al., 2003) and is around 60% in China (Zhang, et al., 2003). The ischemic stroke is more common than the hemorrhagic stroke.

In China, the incidence of ischemic stroke is rising over the years gradually (Wang, et al., 2001; Zhang, et al., 2003; Li, et al., 2008) and the rise has been associated with the fast development of economy and thus changes in diet structure and life style (Xu, et al., 2008a). The prevalence of stroke is different in different areas, being higher in north China than in south China. The highest of incidence of stroke has been found in north-eastern China (486/1000000) and the lowest in South China (136/100000); and the incidence of northern China ranked in the medium level (He, et al., 1995). Furthermore, ischemic stroke was suggested to be more significant in male than in female (Cheng, et

al., 1995). It was reported in an epidemiological investigation that from 1991-2000, the mortality rate of ischemic stroke per 1631 Chinese was 16.9% (Zhang, et al., 2003).

In Hong Kong, it was found that the overall 5-year accumulative evaluation of death, re-stroke and poor prognosis were 31% and 41% respectively (Man, et al., 2009), from a long term study following an investigation of acute ischemic stroke patients with concurrent lesions.

Presently, the high risk of ischemic stroke demanded effective treatment methods. Prevention of stroke, particularly of ischemic stroke, is still considered to be the most effective approach to reduce the danger and risk in these patients.

1.2 The influence of hypertension on stroke

Hypertension is the most common cardiovascular disease, and can lead to severe damages in many vital organs of the body. Stroke and heart diseases are considered to be the two common diseases related to hypertension. Ninety percent of hypertension is primary hypertension. The primary hypertension is induced by polygenic inheritance factors combined with environmental factors such as stress, but the pathogenesis is unclear (Takahashi and Smithies, 2004).

Hypertension is one of the most important risk factors of stroke in the general population. One of the reasons is the formation of blood clots within the blood vessels in hypertensive patients (Sacco, et al., 1991; Barone, et al., 1992; Lassen, 1996) which results in loss of blood supply to the brain tissue. Another reason is that hypertension

increased the incidence of ischemic stroke (Li, et al., 2005; Liu, et al., 2005). The most possible outcome of hypertension is stroke in China but coronary heart disease in most western counties.

Evidences from China and foreign countries revealed that both isolated systolic hypertension (ISH) and isolated diastolic hypertension (IDH) are independent risk factors of stroke. The onset and prognosis of stroke are closely associated with the severity and history of hypertension. For instance, higher blood pressure predisposes higher incidence of stroke (Fang, et al., 2006).

An investigation on the relationship between the control of blood pressure and brain stroke showed that the prevalence of stroke in controlled hypertensive patients was 25.7% lower than in patients with no hypertensive control. The better the blood pressure control, the lower the incidence of stroke. In particular, the occurrence of ischemic stroke was decreased by 19.1% (Ru, et al., 2008). This therefore indicates that controlling blood pressure would help decrease the incidence of stroke. However, it remains unclear how elevated blood pressures are linked to increased cerebral damage in hypertension.

1.3 Chronic brain ischemia and ischemic stroke

Chronic brain ischemia is frequently found in long or short term patients who suffer from cerebrovascular changes, before the onset of ischemic stroke and usually after periods of hypertension in the Chinese population (Zhang, et al., 2009a). The commonest cause of cerebrovascular diseases is cerebral arteriosclerosis, including

atherosclerosis, amyloid angiopathy and precapillary arteriole arteriolosclerosis; atherosclerosis could account for most of the cases (Levi, et al., 2009). The cerebral arteries are narrowed down during the development of arteriolosclerosis, and this results in the deficiency of blood supply to brain cells. Gradually, the cerebral arteries are completely blocked by the blood clots; stroke would happen in the area which lacks blood supply. Hypertension is an important risk factor which induces the generation and development of arteriosclerosis (Yamori, et al., 1976a). For example, in Yamori et al's study, some 90% of patients with a long history of hypertension usually had arteriosclerosis. It has been accepted that arteriosclerosis combined with hypertension would enhance the severity of brain ischemia.

1.4 The study of animal model of hypertension

Currently, many animal models have been used to study different types of human hypertension (Morris, 1984; Wang, et al., 1998; Ye, et al., 2002). The spontaneously hypertensive rat (SHR) is considered as the best animal model of human primary hypertension, and has been extensively used to study cardiovascular and cerebrovascular diseases (Pinto, et al., 1998; DeLano, et al., 2006; Panico, et al., 2009). The SHR model was produced in 1963 by Okamoto through selective breeding of Wistar-Kyoto (WKY) rats with high blood pressure (Okamoto and Aoki, 1963). Blood pressure of SHR is slightly higher than normal around 5-6 weeks old but keeps rising gradually. The systolic pressures may reach the peak between 180 and 200 mmHg in the adult (Conrad, et al., 1995). Similar to the pathogenesis of hypertension in humans, SHR also has polygenic inheritance characteristics (Takahashi and Smithies, 2004).

Previous researches have shown that the rising process of blood pressure in SHR shared similar pathological characters to those in humans. Due to the continuous strengthening of vascular resistance, the renin-angiotensin system in the kidney is activated. This is a process that leads to advanced stage of blood pressure increase (Kodavanti, et al., 2000). Suffering from the continuous development of hypertension, hypertensive rats demonstrated complications similar to human patients with hypertension, such as cerebral apoplexy, myocardial injury and kidney sclerosis (Bing, et al., 1995; Conrad, et al., 1995). Because the damage results in brain function decline, SHR is often used as the ideal animal model to study the mechanism of brain damage resulting from hypertension (Amenta, et al., 2003). Likewise, this model is especially suitable for screening anti-hypertensive agents, such as candesartan which is used in nerve protection in ischemia hypertensive rats (Lu, et al., 2005). Because there are many similarities in development and progress of the hypertension disease between humans and SHR, SHR is an ideal model for our study which aims to evaluate the brain damage resulting from hypertension.

1.5 The study of animal model of chronic brain ischemia

Permanent bilateral common carotid artery occlusion (2-vessel occlusion, 2VO) in rats has been used extensively to study chronic brain ischemia (Ni, et al., 1995; Plaschke, et al., 2001; Kozhechkin, et al., 2009). After the blood vessels were occluded, the forebrain would lack blood supply. With long term low perfusion, animals gradually showed dysfunction in cognition. This model was therefore also used to study vascular dementia (Ni, et al., 1994).

It is known that the cerebral vascular system of rats is generally similar to the human in structure system, which is composed of the carotid and vertebral arteries systems (Lee, 1995). The vessels from the two systems anastomose at the basal forebrain, forming the circle of Willis. This anastomosis supplies blood to the brain areas when one or several blood vessels has been blocked. The circle of Willis although useful, can not prevent acute damage after bilateral common carotid artery occlusion (Sarti, et al., 2002). So the model of permanent bilateral common carotid artery occlusion is the most suitable model to study the stroke phase and subsequent chronic brain ischemia. Subsequent chronic brain ischemia means that the cortex and hippocampus can only get 60-75% of the normal blood flow (Otori, et al., 1997).

It was found that the time of pathologic changes was different in a number of studies of chronic brain ischemia (Pappas, et al., 1996). Pappas et al reported that neurons of hippocampus showed degeneration and immunological activation after a few weeks with permanent bilateral common carotid artery occlusion and learning and memory worsened progressively in the rat. In another study, the immunological activity of glial fibrillary acidic protein (GFAP) increased and peaked at 30 days after permanent bilateral common carotid artery occlusion. This finding showed that the permanent bilateral common carotid artery occlusion model caused progressive damage to glial cells as well (Nanri and Watanabe, 1999).

Ni et al (Ni, et al., 1995), on the other hand, reported that after one month of permanent bilateral common carotid artery occlusion, the cortex and hippocampus showed no changes in general structure or neuron loss under the light microscope. Then 4 months

later, hippocampus neurons were degenerative, followed by glial cells activation. At 7 months, there was neuron loss and the cortex degenerated extensively and became atrophied. Bennett et al thought the loss of neurons was related to cell apoptosis (Bennett, et al., 1998). Besides the changes in the cortex, it was found that myelin disintegrated as well and astrocytes were in a hyperproliferation condition (Lee, et al., 2006a). These studies, nevertheless, were only focused on the instant and subsequent effect of stroke and did not take into consideration of hypertension.

1.6 The study of animal model of stroke

Stroke-prone spontaneously hypertensive rat (SHRsp) is an animal model in which stroke occurs spontaneously. Named by International Society of Hypertension (Okamoto, et al., 1986), it is a subunit of SHR. SHRsp was inbred from rats that usually died of stroke naturally (Yamori, et al., 1976b). The blood pressure starts to increase at the age of 6 weeks, reaches 200 mmHg at 10-15 weeks, peaks (230-250 mmHg) at 5-6 months, and falls at 7 months. SHPsp have a mean life span from 9 months (male) to 12 months (female); they not only have hypertension but 80% of them would suffer from natural stroke. According to the classification of the pathology of stroke, SHRsp develop cerebral infarction while showing little hemorrhage (Amerg, 1982).

1.7 The application of functional magnetic resonance imaging

Functional magnetic resonance imaging (fMRI) is an imaging technology to study brain function. It makes use of the blood-oxygenation-level-dependent (BOLD) imaging

technology to study cerebral blood flow (Bock, et al., 1998). It has been reported that, when the brain functional area was activated by stimuli, the blood flow increased at activated cortical local areas, leading to local oxyhemoglobin increases and deoxyhemoglobin decreases. The proportion between the oxyhemoglobin to deoxyhemoglobin changes constitutes the signals for MRI. According to this mechanism, the activity induced by various stimuli in different areas of the brain can be observed with fMRI (Cornelissen, et al., 1997; Kammer, et al., 1997; Detre and Wang, 2002).

It has been demonstrated that cerebral blood flow (CBF) of a brain area is closely related to its glucose intake and glucose metabolism (Baron, et al., 1982; Gross, et al., 1987), and the local glucose uptakes can be used as a reliable indicator of the metabolic activity in the area (Shockley and LaManna, 1988). Overall, the increases of cerebral blood flow and brain metabolism may reflect the improvement of neuron functional activity (Shulman and Rothman, 1998; Heeger and Ress, 2002). fMRI could therefore indeed reflect indirectly the changes of metabolism and function of local brain cells by detecting the changes of CBF and oxygen saturation.

fMRI has the advantages of precise time and spatial resolution, non-invasiveness, good repeatability, and causing no radiation, and it can depict anatomical and functional images simultaneously (Logothetis, et al., 1999; Kim, et al., 2000). Therefore, it is now widely applied to almost all areas of neurosciences, such as perception (Katata, et al., 2009), motor activity (Calautti, et al., 2001), learning and memory (Shaywitz, et al., 1999), emotion and cognitive (Koshino, et al., 2005). Presently, there is no report on

chronic cerebral ischemia research (e.g. hypertension with experimental stroke and hypertension with stroke) using BOLD fMRI.

Generally, short stimulation and resting are used in fMRI experiments (Zhang, et al., 2007). The stimulus "on" and "off" process is known as an experimental cycle, and multi-cycles are commonly used. However, functional diagnosis and the study of brain mechanisms in brain disease are two different aspects (Turner, et al., 1998; Golay, et al., 2000). In the clinical study on ischemic cerebrovascular diseases fMRI is applied mainly in early diagnosis of ischemic stroke, and in the monitoring after stroke (Demougeot, et al., 2002; Abe, et al., 2003). BOLD fMRI are rarely used until recently when research showed that BOLD fMRI could display the changes in the recovery process of cortical areas in patients with cerebral apoplexy (Heller, et al., 2005).

1.8 The neuroprotective effect of Pien Tze Huang

In this study, we also evaluated the ability of Pien Tze Huang (PTH) in preventing chronic brain ischemia and stroke. PTH is an ancient but precious traditional composite Chinese medicine, with a history of over 500 years. Now, it has been authorized to be manufactured exclusively by Zhangzhou PTH Pharmaceutical Co., Ltd., in Fujian province. The formula and producing process of PTH has been highly protected as a national secret. The published main components of PTH include Radix Notoginseng (85%), snake's gall (7%), calculus bovis (5%) and natural musk (3%) [http://www.zzpzh.com/pzh/n-tegong.htm].

PTH exhibits a wide range of pharmacological effects and functions, such as anti-inflammation, detoxification, analgesia and anti-edema (Lin, et al., 1985; Zhao and Pan, 2006; Meng and Gu, 2008). It has been widely documented in the traditional Chinese medicine literature that Radix Notoginseng and snake's gall are anti-inflammatory and are used for detoxifications. Calculus bovis is also anti-inflammatory ("taking off the heat") while natural musk is for anti-edema. PTH has also been extensively applied in China for the treatment of viral hepatitis, tumor growth, and cancer pain, and for reducing the chemotherapy-associated toxicity (Xu and Yan, 2003). Recently, we observed that PTH could significantly reduce the survival of neuroblastoma cells (SH-SY5Y) *in vitro* (Lü, et al., 2009), indicating that PTH may exert other actions. However, so far, there is little direct evidence on the neuroprotective effect of PTH.

The most important component of PTH is Radix Notoginseng, the root of *Panax notoginseng*; Panax notoginseng saponins (PNS) is the main active substance, making up to 12% of the PTH contents, and contains various monomers such as ginsenoside Rb1, Rg1 and panaxatriol saponin (PTS) (Dong, et al., 2003). A large number of studies showed that the ingredients of PNS and its monomer have many functions on vessels and perhaps on nerve protection. Musk is the secretion of the gland situated between the umbilicus and genitalia of a mature male musk deer. Muscone is the main active component of musk (Sun, et al., 2005), Calculus bovis is the stone in the gallbladder, bile duct or hepatic duct of the ox. Its main contents include bilirubin, taurine, cholic acid, deoxycholic acid and cholylglycine. Snake's gall contains different kinds of biles; the principle components include taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), lithocholic acid and free bile acid.

(1) Protection of vascular endothelium

Plasma endothelin (ET) is synthesized mainly by endothelial cells and is a tripeptide which promotes vascular contraction of blood vessels (Yanagisawa, et al., 1988; Motte, et al., 2006). Endothelin aggravates vascular endothelial dysfunction and causes vascular remodeling by activating oxidative stress to blood vessel walls and vascular smooth muscle cell (VSMC) proliferation (Schiffrin, 2005). Proliferation of vascular smooth muscle cells is the common pathological feature of diseases such as hypertension and atherosclerosis (Zhu, et al., 1998). Hyperlipidemia can also cause atherosclerosis (Tannock, 2008). Plasma endothelin is at high level in patients with acute cerebral infarct, and its increased presence has been positively correlated with the size of the focal cerebral infarction (Greenberg, et al., 1992). PNS can effectively reduce plasma endothelin of cerebral infarction patients, mitigate the damage to ischemia brain, inhibit the proliferation of vascular smooth muscle cell, and reduce blood-lipid (Kenarova, et al., 1990). It also protects endothelia, thereby reducing the risk of atherosclerosis (Zhou, et al., 2003).

Hypertension can also cause endothelial injury and induce the secretion of plasma endothelin (Miyauchi, et al., 1989). Muscone can reduce plasma endothelin in stroke-prone renovascular hypertensive rats (RHRSP); it might be protective to vascular endothelial cells, and to some extent can amend vascular remodeling caused by hypertension (Li, et al., 2006a). Muscone can therefore delay the development of hypertension and the injury to the brain caused by hypertension. Taurine, another ingredient in muscone, can also inhibit vascular smooth muscle cell proliferation

induced by endothelial injury through inhibiting calcium in-flow and reducing the calcium content.

(2) Effect on metabolism

Almost 100% of the energy in the central nervous system comes from the oxidation of glucose. Glucose metabolic disorder is one of the main reasons of central ischemic injury (Zhang, et al., 2001). In the cases of anoxia, anaerobic glycolysis increases, and lactate increases. The raised lactate is an index of oxidative metabolism disorders (Eliash, et al., 2005). Calculus bovis can increase the glucose reserves in the extracellular fluid of hippocampus of SHR, reduce lactate content, and thereby improves the energy metabolism and protects the brain tissue (Cao, et al., 2008).

(3) Effects on clearance of free radicals

When cerebral ischemia occurs, the brain produces lots of free radicals, which can attack membranes composed of unsaturated fatty acid, leading to lipid peroxidation and overproduction of the end-product malondialdehyde (MDA). Damage of the membrane structure and changes in the structure and function of biological macromolecules occurs within the cell causing cell death (Ceballos-Picot, et al., 1992). Superoxide dismutase (SOD) is the only enzyme which can remove superoxide anions (O_2^-) in our body, so it is the key enzyme of antioxidant enzyme system (Guemouri, et al., 1991). The content of MDA and SOD activity can indirectly reflect the degree of cell injuries and the free radical scavenging capacities (Clarkson, 1995; Karlsson, 1997; Rudnicki, et al., 2007).

PNS and its monomer Ginsenoside Rgl can protect the activity of endogenous SOD and reduce MDA (Kenarova, et al., 1990; Han, et al., 1999). Muscone can improve the contents and activities of SOD and reduces the contents of MDA in the brain and the plasma of cerebral ischemia in rats (Sun, et al., 2009). The two main components of muscone, bilirubin and taurine, especially bilirubin has defined antioxidant effect (Stocker, et al., 1987). It can significantly improve the activities of SOD in hippocampal tissue of SHR and clear away free radicals, resist lipid peroxidation and reduce the MDA contents. Hence these active substances can maintain the stability of brain tissue and endothelial cell biological membrane, increase the anoxia tolerance level of brain and reduce the damage to blood vessels and the brain.

(4) Effect on intracellular calcium homeostasis

In the early ischemia, abundant excitatory amino acids (EEAs) are released into extracellular fluid of the brain, activating Ca^{2+} voltage-dependent and N-methyl-D-aspartic acid (NMDA) receptors related channels; this attenuates Ca^{2+} flow, causes Ca^{2+} overload inside the cells and triggers a series of biochemical reactions that is Ca^{2+} dependent, resulting in various changes in functions and structure of the cells (Zuccarello and Anderson, 1989).

Both PNS and taurine have calcium channel blocking functions, thus they can decrease calcium overload of damaged nerve cells (Nah, et al., 1995; Han, et al., 1999). Muscone can reduce the contents of EEAs in brain tissues and the expression of NMDA receptors. It can also inhibit Ca^{2+} channels which is voltage-dependent and NMDA related

receptors, reduce the influx and prevent the overload of Ca^{2+}, hence reducing the injury to cell resulting from a series of Ca^{2+} dependent responses (Liang, et al., 1996).

(5) Improvement of the cerebral circulation

Previous experiments confirmed that PNS can expand brain blood vessels and improve the cerebral circulation (Nah, et al., 1995; Han, et al., 1999). Other studies had pointed out that PNS, muscone and taurine all might be related to the Ca^{2+} channel blockage by these substances and hence might reduce the damage of brain caused by cerebral edema.

(6) Inhibition of platelet aggregation

The effective ingredients of PNS, ginsenoside Rgl and PTS could inhibit platelet aggregation in various ways, such as improving cyclic adenosine monophosphate (cAMP) contents in platelets and reducing production of thromboxane A_2 (TXA_2) (Xu, et al., 1998), and hence making the blood thinner. Musk and taurine can inhibit platelet aggregations via various mechanisms, such as the lowering of blood-lipid, hence reducing hypercoagulation and delaying the developments of atherosclerosis (Zang, et al., 2001).

(7) Protection of the injured neurons and promotion of the neurological recoveries

Musk can significantly reduce the volumes of rat brain infarction and the number of neurons at half dark zone around the infarct area (Jiang, et al., 2007). At the same time, Musk also can significantly increase the number of astroglia, and enhance nestin and

glial fibrillary acidic protein (GFAP) expression around the infarction. All the data suggest that musk can promote reactive gliosis in ischemic area, protect the neurons from injuries, induce proliferations of neural stem cells, and increase revascularizations.

(8) Function of reducing blood pressure

PTS could reduce blood pressure and stroke incidence in stroke-prone renovascular hypertensive rats (RHRSP) (Zhao, et al., 2006). PNS dosage-dependently reduces systolic and diastolic blood pressure with gentle and lasting effects. Taurine can also reduce the blood pressure of SHR and delay the progressive changes to hypertension (Gao, et al., 2008). Snake's gall, of which the main component is also taurine, has the effects of lowering the blood pressure. The mechanism of anti-hypertension by PNS and taurine is related to expansion of blood vessels and the blockage of calcium channels (Nah, et al., 1995; Han, et al., 1999). Muscone also has a certain action to lower blood pressure.

(9) Anti-aging and anti-dementia function

PNS can improve the levels and activities of acetylcholine transferase (ChAT) and synaptophysin, protect and improve the central cholinergic system, and ameliorate the symptoms of aging and dementia. PNS, ginsenoside Rb1 and ginsenoside Rg1 can significantly enhance the learning and memory abilities of mice, and this may be correlated with the regulation of hippocampal synaptic activities, ATPases and calcineurin (Jin, et al., 1999).

Totally, many active compositions of PTH are known to protect nerves and vessels, dilate blood vessels, improve cerebral blood flow, resist oxidation, lower blood pressures, and inhibit neurotoxicity. This suggests that PTH might also have protective effects on the brain as a whole, and provides new ideas for further development of PTH.

1.9 Summary

Being one of three dangerous diseases in the world, the prevalence of stroke is going up with the expansion of the aging population of the world. However, effective methods and treatments are in hand to control this disease. It appears that controlling the risk factors of stroke is the most effective approach to reduce the onset of stroke and attenuate the damage of stroke in patients. The two most dangerous and important factors of stroke are hypertension and cerebral arteriosclerosis, which when occur at the same time in brain ischemia would increase the incidence of stroke and severity of the injury. To some degree, the incidence of stroke may be reduced by controlling hypertension and arteriosclerosis.

However, the pathology of the brain suffering from combined hypertension and cerebral arteriosclerosis is not well understood. No animal models have been used to imitate this pathological. It is well known that spontaneously hypertensive rat is an ideal model to investigate human primary hypertension, and the model of 2-vessel occlusion has been extensively applied in chronic brain ischemia research. Unfortunately, there are few reports on 2-vessel occlusion of SHR.

PTH is a famous, expensive traditional Chinese medicine for treating liver disease and tumor. PTH is mainly composed of Radix Notoginseng, snake's gall, calculus bovis and natural musk, which exert a significant protective action on neurons and blood vessels. Hence it is possible that PTH also has a function in protecting neurons and blood vessels. Yet no reports had been found to study this protective role of PTH, both in clinical and basic researches.

In the present study, 2-vessel occlusion was performed on SHR. This produced an animal model of hypertension combined with chronic brain ischemia. With this model, it was possible to study the combined effects of these two processes on brain tissue. In addition, the model would allow a study of the protective role of PTH in neurons and blood vessels in such brains.

2.0 Aims of this study

(1) To assess the risk factor and prognosis in ischemic stroke patients from mainland China.

(2) To establish an animal model of hypertension combined with chronic brain ischemia.

(3) To delineate pathological changes in the hippocampus and cerebellum during different periods of hypertension combined with chronic cerebral ischemia.

(4) To explore the neuroprotective effect of PTH in hypertension combined with chronic brain ischemia.

(5) To explore the neuroprotective effect of PTH in stroke.

Chapter 2 Clinical analysis of risk factors and prognosis of ischemic stroke patients in mainland China

2.1 Introduction

Stroke is one of the commonest diseases that threaten the health of the world. It has been associated with high incidence and high mortality (Thorvaldsen, et al., 1995; Feigin, 2005). In China, more than 60% of these strokes are ischemic stroke (Zhang, et al., 2003) and hypertension has been known to be an independent and yet the most crucial factor for stroke (Fang, et al., 2006).

With the advancement of sciences and economics, China faces a problem of an increasingly aging population. The life style, diet and pressures from the society have changed significantly. The present section analyses the clinical data from ischemic brain stroke patients in south and north China between May 2005 and June 2007. The aim is to study the relationship and prognosis between ischemic brain stroke and hypertension in the recent years to provide useful information to the prevention the onset of ischemic brain stroke.

2.2 Materials and methods

2.2.1 Clinical cases

From May 2005 to June 2007, 290 and 82 patients of ischemic stroke were respectively recruited in the No.1 Hospital of Qinhuangdao in north China and the Third Affiliated

Hospital of Sun Yat-sen University in southern China. All patients fulfilled the diagnosis standard of brain stroke issued by WHO MONICA (Multinational Monitoring of Trends and Determinants in Cardiovascular Disease) Project (Truelsen, et al., 2003), and all patients' conditions were confirmed by magnetic resonance imaging (MRI) examination.

2.2.2 Methods

Retrospective investigation was carried out on patients with ischemic brain stroke: personal data, clinical manifestation, history of hypertension, other diseases patients were suffering, risk factors related to brain stroke, accessory test, MRI examinations, treatments and outcomes. All studies and data were taken with patients' consent and the consent of the affiliated ethical committee of the respective hospital.

2.2.3 Statistical analysis

All data are presented as mean ± standard deviation (SD). Pearson Chi-square test was performed using SPSS 16.0. $p \leq 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Age structure of ischemic stroke patients

Amongst the randomly selected 372 cases of ischemic stroke individuals, the youngest was 20 years old and the oldest was 91, with the average at 62.38 ± 12.31 (mean ± SD) years old. Patients were grouped by intervals of 10 years. There were 3 patients (0.81%)

in the group of less than 30 years old, 15 (4.03%) of 30-39 years old, 31 (8.33%) of 40-49 years old, 100 (26.88%) of 50-59 years old, 105 (28.23%) of 60-69 years old, 94 (25.27%) of 70-79 years old, 22 (5.91%) of 80-89 years old and 2 (0.54%) of more than 90 years old. According to the table below, patient number increased with the age and reached the high scores in the groups of 50-59, 60-69 and 70-79, and then declined in patients of more than 80 years old (Table 2.1). Over 80% of patients were aged between 50 and 79.

2.3.2 Gender of ischemic stroke patients

There were 245 (65.86%) male patients and 127 (34.14%) female patients. Male to female ratio was 1.93:1.

2.3.3 Hypertension in ischemic stroke patients

There were 254 ischemic stroke patients (68.28%) who had hypertension and 118 patients (31.72%) who had other risk factors, including diabetes, hyperlipidemia, coronary disease, smoking and alcohol consumption (Table 2.2).

2.3.4 Prognosis of ischemic stroke patients who had hypertension

For all the patients, the same treatments or regimens were given and the conditions of the patients were showed in Table 2.2 when they left the hospitals. Among the total 372 cases, 8 people died (2.15% case fatality rate); 7 of them (87.5%) died of respiratory and circulatory failure caused by brain hernia and 1 died of severe infection in the lungs.

When patients were divided into hypertension and non-hypertension groups as showed in Table 2.2, the difference in the number of the 2 groups was significant (p = 0.05). Therefore, we concluded that there were significant effects of hypertension on the outcomes of the patients' conditions.

2.4 Discussion

Age is an unchangeable risk factor to stroke. The present study showed that the number of patients increased with age. For instance, the numbers of patients suffering from stroke increased very fast at 50 to 79 years old and then after 80 years old, the numbers declined. This trend might reflect the development of certain risk factors such as hypertension, heart disease and diabetes after 50 years of age.

Our results showed that the number of male patients almost doubled the number of female patients by a ratio of 1.93:1 (65.86% vs 34.14%). This result is similar to the finding in a previous study fifteen years ago (Cheng, et al., 1995). The difference in male to female might be due to differences in diet, alcohol and cigarette consumption, or prevalence of hypertension among gender (Xu, et al., 2008a). The difference between male and female apparently remained the same between the previous study 15 years ago and our present study.

The present study revealed that more than two thirds (68.3%) of ischemic stroke patients had a history of hypertension before the onset of stroke, while about one third (31.7%) of patients did not have hypertension before (Table 2.2). Compared with patients without hypertension, stroke patients with hypertension had significantly worse

prognosis ($p = 0.05$). This indicates that hypertension is relevant to ischemic stroke as an important causal factor, and it might also lead to significantly worse outcomes. This finding is consistent with previous studies which proposed that hypertension was a high risk factor of stroke in China (Fang, et al., 2006). Hypertension not only increased the incidence of stroke but also enhanced the severity of stroke (Li, et al., 2005; Liu, et al., 2005). Therefore, it would be possible to lower the incidence of stroke via controlling blood pressure (Ru, et al., 2008). Previous studies have confirmed that hypertension could promote arteriosclerosis and stroke (Ueda, et al., 1992). Indeed, changes were found in hypertensive patients including the damage of endovascular epithelial cells, aggregation of blood platelets, dysfunction of blood coagulation and higher blood viscosity. All these factors could induce or enhance ischemic stroke. However, the real mechanism of hypertension on stroke is still not clear.

Amongst the 8 deaths, 7 died of central respiratory and circulatory failure caused by hernia of the brain. One died of severe pulmonary infection. These results suggested that the main cause of death was severe infection, not stroke. This finding was consistent with some Chinese publications (Huang, et al., 1999). The death caused by ischemic stroke may be related to the damage of automatic nervous center, resulting in organ dysfunction, blockage of airway and other serious complications.

To sum up, prevention and control of hypertension is important. At the time of stroke, proper treatment, such as controlling blood pressure, decreasing encephalic pressure, preventing and controlling infection are all crucial.

2.5 Conclusions

(1) Ischemic stroke is a disease with high disability rate and case fatality rate. Our hospital records show that there were almost twice more male patients than female patients.

(2) Hypertension is a pivotal risk factor.

(3) Hypertension increases the severity of stroke and leads to significantly poorer outcomes.

Table 2.1 Age of ischemic stroke patients

Age (years)	Cases	Percentage
<30	3	0.81
30~39	15	4.03
40~49	31	8.33
50~59	100	26.88
60~69	105	28.23
70~79	94	25.27
80~89	22	5.91
>90	2	0.54
Total	372	100

Table 2.2 Prognosis after ischemic stroke

		Prognosis			
Patients risk factor	Cases	Death	Severe disability	Mild disability	Substantial improvement
Hypertension	254	7	92	114	42
Non-hypertension	118	1	29	58	29
Total	372	8	121	172	71

Pearson Chi-square $F = 7.793$, $p = 0.05$

Chapter 3 Proof of ischemia in the rat model after permanent occlusion of bilateral common carotid arteries by functional magnetic resonance imaging

3.1 Introduction

In any experimental work, it is important to prove that the technique actually worked. In this study, therefore, it was important to prove that the rats with surgically occluded bilateral common carotid arteries (2-vessel occlusion, 2-VO) in fact represented a model of cerebral vascular occlusion or ceased neural activities. Although the bilateral common carotid arteries (CCA) occlusion model had been performed in experiments for many years and in fact were still performed recently (He, et al., 2008; Molnar, et al., 2008; Xu, et al., 2008b; Kozhechkin, et al., 2009; Sivilia, et al., 2009; Ye, et al., 2009; Zhang, et al., 2009b) and that biochemical changes (Molnar, et al., 2008; Xu, et al., 2008c; Sivilia, et al., 2009; Ye, et al., 2009) as well as psychiatric changes (He, et al., 2008) had been documented, the *in vivo* events inside the brain are still unknown.

Functional magnetic resonance imaging (fMRI) is an imaging technology which can test the cerebral blood flow and the functional changes of metabolic activities. It is based on blood-oxygenation-level-dependent (BOLD) and contrast-enhanced imaging (Bock, et al., 1998). The blood flows of different brain areas correlate with cortical activation and metabolic activities of those areas. fMRI has features of precise spatial and time resolution, non-invasiveness and repeatability, being non-radiation, and the simultaneous formation of anatomical and functional images (Logothetis, et al., 1999). Thus, fMRI is

a good technical approach to detect the changes of cerebral blood flow in different brain regions.

In this work, we reported the sequence of vascular oxygen changes (BOLD image) by employing fMRI. We gave the animals stimulation to the tail and recorded the fMRI. This method had been pioneered in our laboratory and in small animals (Fang, et al., 2005; Lee, et al., 2006b; Wai, et al., 2007; Zhang, et al., 2007).

3.2 Materials and methods

3.2.1 Material

Ketamine, xylazine, amoxicillin and buprenorphine were purchased from Alfasan Group Companys (Woerden, Holland); 5-0 Sterile polypropylene suture was purchased from ETHICON Inc. (Somerville, NJ, USA). All other chemicals used were of analytical grade and were obtained commercially.

3.2.2 Animals

Approval on animal experimentation had been obtained from the Animal Experimentation Ethics Committee (AEEC), Chinese University of Hong Kong (CUHK). The animals were supplied by the Laboratory Animal Services Centre (LASC) of CUHK.

All animals in this project were fed in groups of 4-5 per cage and the rats were kept in an air-conditioned room maintained at 22 ± 1 °C, with a 12-hour light-dark cycle. Standard food pellets and tap water were provided regularly throughout the experiment.

Male spontaneously hypertensive rats (SHR) of 5-6 months old, 197 in total, and 122 age-matched normotensive Wistar Kyoto (WKY) rats, were used. (Note: the numbers of SHR and WKY as described here were used for experiments in this and the following Chapters). Rats were randomly divided into four groups, two chronic brain ischemia groups and two sham operation groups, which were named respectively WKY chronic brain ischemia group (WKY-ischemia), SHR chronic brain ischemia group (SHR-ischemia), WKY sham operation group (WKY-sham) and SHR sham operation group (SHR-sham). The bilateral CCA were occluded in animals to cause chronic brain ischemia. As a control, the rats of the sham groups were operated in the same way as the chronic brain ischemia groups, but without occlusion of bilateral CCA.

3.2.3 Preparation of the animal model

The model of 2-vessel occlusion was modified from the previous publications (Watanabe, et al., 1996; Murakami, et al., 1997). The bilateral CCA were cut off to avoid incomplete occlusion of bilateral CCA and the reestablishment of blood flow. The operation was described in more detail as followings:

Disinfected equipments and tools (forceps, scissors, clamps, needles, knives, cotton, gauze and threads) were used. Rats were anaesthetized by intra-peritoneal injection with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), and placed in a supine position. The front of the neck was shaved and the skin was disinfected with 70% ethanol. A cut was made in the middle line for 1.5-2 cm. Subcutaneous tissue was separated to exposure the common carotid arteries which were located beside the thyroid

gland. Tissues around the arteries were isolated such that the accompanying nerve fibers were not injured. The distal and proximal parts of the common carotid artery were ligated with a 5-0 sterile polypropylene suture. Hence a gap of about 0.5 cm was left between two ends. The CCA was transected between the two ends with eye scissors. Muscles and skin were sutured and amoxicillin was applied to the wound for local anti-infection and analgesic Buprenorphine was used after surgery for a total of three days (0.01-0.05 mg.kg-1 s/c, 2 times per day).

3.2.4 Functional magnetic resonance imaging

From the 4 groups of rats (WKY-operation, SHR-operation, WKY-sham and SHR-sham), 6 rats from each group were used for fMRI study. After the ischemic surgery, at 15, 30 and 60 minutes, each rat was given a fMRI test to evaluate the changes of blood flow of different brain regions. After the test, the rats were allowed to recover; they were closely monitor and would be used used for the other experiments of this project.

For fMRI, firstly, the rats were put in a prone position and symmetrically fixed. After the appropriate coils were put gently onto the head, MRI scan was performed. Then the tails of the rat were stimulated with a 500 gram weight, and simultaneously a scan was performed and the functional changes of the brain recorded. The detailed procedure of fMRI is described as follow:

For the tail stimulation, we used an "off-on" stimulation mode. Each stimulation session was performed according to the following sequence: a baseline scan of 4 seconds, a rest period of 10 seconds (off), followed by a 10 seconds of stimulation (on) by the weight.

For each stimulus, the "off-on" cycle was repeated three times. For fMRI data acquisition, an echo planar imaging (EPI) pulse sequence was used (TR = 2000 ms, TE = 33 ms, slice thickness = 0.8 mm, slice gap = 0 mm, FOV = 60 mm, matrix = 64*64, NSA = 1). The images were scanned in the coronal plane with a slice number of 12. In total, 64 acquisitions were obtained for each fMRI experiment. The fMRI images were analyzed with ViewForum workstation (Phillips Medical System, Best, Netherlands).

3.3 Results

3.3.1 The operative mortality

During and after the operation, some animals died. The operative mortality was 26.42% in SHR-ischemia group and 22.89% in WKY-ischemia group. Rats that survived the first post-operative 48 hours would stay alive. There was no death in the WKY-sham and SHR-sham groups.

3.3.2 Functional magnetic resonance imaging

When the tail was stimulated, the SHR-sham and WKY-sham rats showed similar activated regions; as well these reactions did not change over time. But the activated brain regions of SHR-ischemia rats and WKY-ischemia rats changed with time, and both showed the similar trends of changes.

The 500g stimulus on both SHR and WKY sham groups triggered a response in the sensory cortex and the cerebellum (Figure 3.1A). In the SHR and WKY ischemia groups,

after 15 minutes of ligation, the BOLD fMRI image decreased in both the sensory cortex and cerebellum (Figure 3.1B). Thirty minutes after the ligation, both regions were devoid of any signals (Figure 3.1C). One hour later, however, the signals returned to some parts of cortex and the cerebellum, but in much lower intensity (Figure 3.1D).

3.4 Discussion

fMRI has the strong features of spatial and time resolution on the changes of blood oxygen and blood flows; it provides the best evidence on the functional activities of the brain (Logothetis, et al., 1999). Previous studies had confirmed that the rat had a very similar distribution of brain blood vessels as that of humans, The cerebral arterial supply is composed of the carotid artery system and vertebral-basilar artery system. When both common carotid arteries are blocked, the lateral brain areas of the rats will get their supply from the vertebral-basilar artery through the circle of Willis (Sarti, et al., 2002). The blood supply of the vertebral-basilar system will show a corresponding reduction due to this compensatory role.

In this work, we were the first to prove unequivocally that the bilateral common carotid arteries ligation in the SHR and WKY rat resulted in a lowering of vascular oxygen, and that this happened 15 minutes after lesion in the cortex and cerebellum. Thirty minutes after the occlusion, an almost total lack of vascular oxygen in the cortex and cerebellum was observed; this might be due to the emergence of a more serious ischemia and the subsequent decompensating state. By 60 minutes later, there was a return of BOLD signals (vascular oxygen level) in the cortex and cerebellum. Thus this experimental

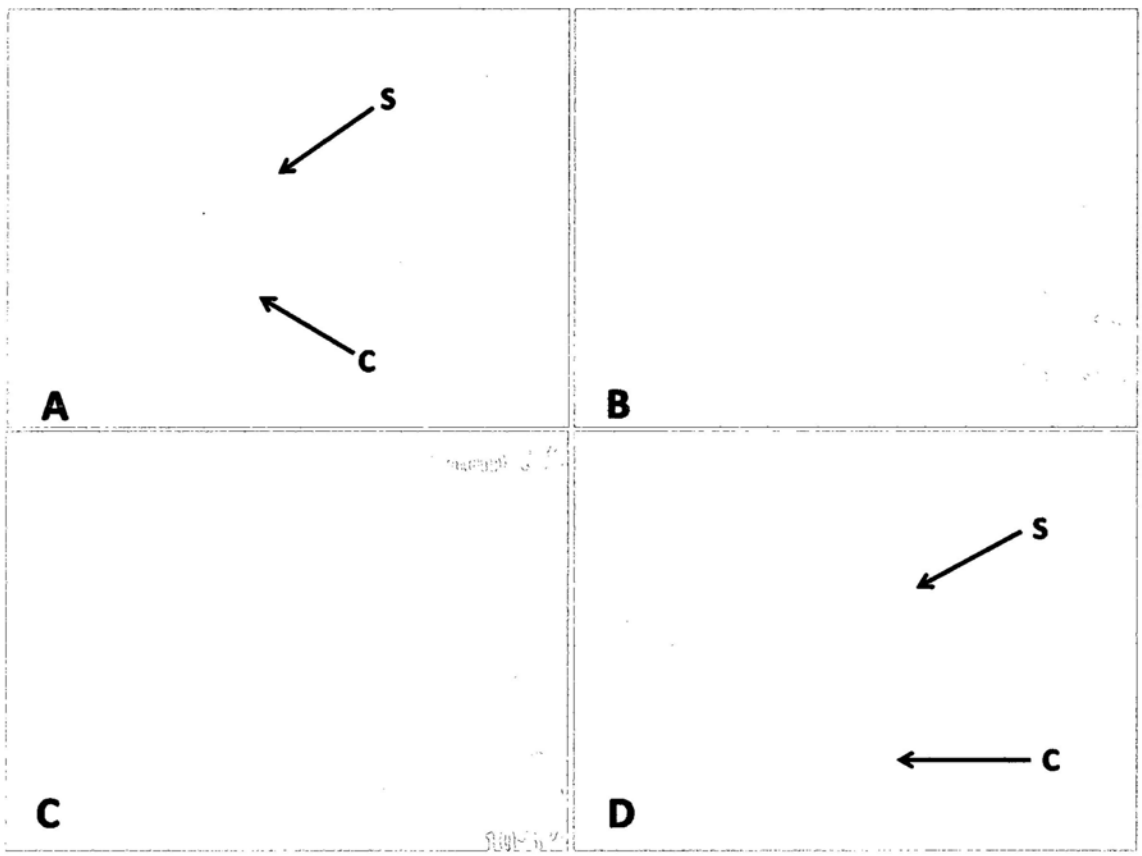
stroke model did subject the cortex to a major ischemia for a period of 30 minutes after the occlusion. However, the stroke period was brief and the long term effect on the model became that of chronic ischemia.

Our experimental results also indicated that the SHR and the normotensive WKY rats had a similar compensatory capacity in the early ischemia period. This may be due to the same distribution patterns of cerebral arteries in these two strains (Ogata, et al., 1976). This may also explain the similar surgical mortalities between the two strains.

3.5 Conclusion

With the permanent bilateral occlusion of the common carotid artery in the SHR and WKY rats, an animal model of brief stroke plus long term chronic ischemia was successfully produced.

Figure 3.1 BOLD fMRI images during rats tail stimulation. (**A)** BOLD fMRI images of SHR and WKY sham groups. S denotes sensory cortex and C denotes cerebellum. **(B)** BOLD fMRI images showing decreased signals upon tail stimulus given 15 minutes after the ligation in SHR and WKY rats. (**C)** Total absence of BOLD image upon stimulus given 30 minutes after the ligation. (**D)** A slight return of signals in both areas upon stimulus 60 minutes after the ligation.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Chapter 4 Pathological changes of hippocampus and cerebellar Purkinje layer in chronic ischemia with hypertension

4.1 Introduction

Based on the pathological characteristics, strokes can be classified into ischemic stroke and hemorrhagic stroke. Approximately 60%-80% of the strokes in the world are ischemic strokes (Akopov and Cohen, 2003; Ovbiagele, et al., 2003). However, the effective treatment for stroke is insufficient at present.

Hypertension is the most important risk factor of stroke; it could increase the incidence and danger of ischemic stroke in patients (Li, et al., 2005; Liu, et al., 2005). Cerebral atherosclerosis is another important risk factor of stroke; it causes cerebral vessels to narrow and the brain tissue would be deprived of adequate blood supply for a long period before the onset of stroke. Approximately 90% of patients with long history of hypertension usually had arteriosclerosis (Yamori, et al., 1976a). Therefore, it is common in patients that chronic brain ischemia is combined with hypertension prior to stroke. It will be important to study the combined influence of these pathologic conditions in order to prevent ischemic stroke.

In clinical and basic studies, most studies have focused on either hypertension or chronic cerebral ischemia only (Nanri, et al., 1998; Yao, et al., 2007), and only few studies were on chronic cerebral ischemia combined with hypertension. Currently, there is no satisfactory animal model to mimic the chronic brain ischemia combined with hypertension in human. The rat model of permanent ligation of both common carotid

arteries (2-vessel occlusion, 2-VO) is an ideal model used by most researchers to investigate the chronic brain ischemia and vascular dementia (Ni, et al., 1994; Kozhechkin, et al., 2009). The cerebral blood vessels of the rat are similar to human being, in respect to the presence of carotid arteries and vertebrobasilar arteries. When both common carotid arteries (CCA) are blocked, the lateral and superotemporal brain areas would derive their blood supply from the vertebrobasilar arteries, so ischemia would happen but not stroke (Sarti, et al., 2002). Spontaneously hypertensive rat (SHR) is considered as the best animal model for human primary hypertension, and has been extensively used to study cardiovascular and cerebrovascular diseases (Pinto, et al., 1998; DeLano, et al., 2006; Panico, et al., 2009). SHR has human like polygenic inheritance characteristics (Takahashi and Smithies, 2004) and the rise in blood pressure in SHR has similar pathological characters to those in humans (Kodavanti, et al., 2000).

This study was aimed to study the combined effect of chronic brain ischemia and hypertension, a knowledge gap in the research on stroke that needs to be addressed. It was to explore the pathologic changes in animals suffering from both conditions. The left and right common carotid arteries of SHR would be ligated and the brain would be examined at different periods after the operation.

4.2 Materials and methods

4.2.1 Materials

Amoxicillin and buprenorphine were purchased from Alfasan Group Company (Woerden, Holland). 5-0 sterile polypropylene suture was purchased from ETHICON

Inc. (Somerville, NJ, USA). ApopTag® peroxidase *in situ* apoptosis detection kit was purchased from CHEMICON International, Inc. (Temecula, CA, USA). Cell death detection ELISA kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). DC protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Superoxide dismutase (SOD) assay kit, catalase (CAT) assay kit and glutathione peroxidase (GPx) assay kit were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals used were of analytical grade and were obtained commercially.

4.2.2 Animals

Sixty male 5-6 months old SHR, and 60 male age-matched Wistar Kyoto (WKY) rats serving as controls, were used in this experiment. Rats were randomly divided into four groups, two chronic brain ischemia groups and two sham operation groups, which were named respectively SHR-ischemia, WKY-ischemia, SHR-sham, and WKY-sham. These animals were used to examine the morphologic changes, antioxidants activity, cell death, and changes in brain and body weights.

4.2.3 Preparation of the animal model and weights measurement

The model of 2-vessel occlusion was modified from the previously published articles (Watanabe, et al., 1996; Murakami, et al., 1997). The bilateral CCA were transected to achieve complete interruption of the CCA. The operation is described in more detail as follows:

Disinfected equipments and tools (forceps, scissors, clamps, needles, knives, cotton, gauze and threads) were used. Rats were anaesthetized by intraperitoneal injection with 10% chloral hydrate (3 mg/kg), and placed in a supine position. The front of the neck was shaved and disinfected with 70% ethanol. A 1.5-2 cm cut was made in the midline. Subcutaneous tissue was separated to exposure the CCA which was located beside the thyroid gland. Tissues around the arteries were isolated care being taken to avoid injuring the accompanying nerve fibers. The distal and proximal parts of the CCA were tightened with a 5-0 sterile polypropylene suture, A gap about 0.5 cm was left between the two ends. The bilateral common carotid arteries were cut off between the two ends with an eye scissors. Muscles and skin were sutured, amoxicillin was applied to the wounds and Buprenorphine was used after surgery for three days (0.01-0.05 mg.kg-1 s/c, 2 times per day). The rats were kept in a warm place for an hour and put back to the cage. After that, animals were fed and observed regularly.

Body weights of all rats were recorded as soon as the operation was completed. The rats were sacrificed on post-operation day 15, 30, and 60 later. The brains were removed and washed with normal saline to remove blood, excess fluid was removed with filter paper, and brain weights of 6 rats in each group were measured (Table 4.1 and Figure 4.1). The ratio of brain to body weight was calculated.

4.2.4 Preparation of brain tissue samples

Brain tissue samples from rat groups sacrificed at all time points were prepared for different assays: Nissl staining, TUNEL in *situ* staining, and SOD, CAT and GPx

activities. The cell death ELISA assay was performed on the samples from the day 15 group only (Table 4.1, Figure 4.1).

For Nissl and TUNEL staining, the rats (4 from each group) were anaesthetized by intra-peritoneal injection with 10% chloral hydrate (3 mg/kg body weight). The thorax cavity was opened, the heart was exposed and was perfused with normal saline at the left ventricle. The fluid was pumped from the left ventricle and flowed out of an cut opening in the right atrium. The blood was cleaned by this way about 250 ml of the normal saline. When the color of liver turned from red to white, the normal saline was replaced by 300 ml of 4% paraformaldehyde (PFA). The perfusion should be fast with the saline but slow with the fixative. The brain was removed and dehydrated through serial ethanol, cleared by xylene and infiltrated with paraffin in the Shandon Pathcentre tissue processor (GMI, Inc., Ramsey, MN, USA) (Table 4.2). The blocks were then embedded in paraffin at 60 °C, sliced into 5 μm sections in coronal position. The sections were bathed in 45 °C water, and then attached onto gelatin-coated slides. Pairs of consecutive sections were selected for staining by the Nissl and TUNEL methods respectively.

For the SOD, CAT and GPx assays, 6 rats from each group were used. The hippocampus and cerebellum were isolated and removed and snap frozen in liquid nitrogen. The samples were stored at -80 °C till analyses.

4.2.5 Nissl staining

Sections were dewaxed in xylene three times for 5 minutes each time, hydrated through absolute, 95%, 80%, and 70% alcohol, 5 minutes each time, and finally hydrated in

distilled water for 5 minutes. Slides were stained in cresyl violet for 5 minutes, then rinsed in distilled water, and soaked in 95% ethyl alcohol for 30 minutes. The slides were then dehydrated through serial alcohol. They were then cleared twice in xylene for 5 minutes and mounted with Permount. When slides were observed under the light microscope and the stained Nissl bodies in neurons would appear red-violet.

4.2.6 TUNEL staining

The apoptotic cells in the hippocampus and cerebellar Purkinje layer were detected by TUNEL staining. The procedure was performed, with modifications, according to the manual from CHEMICON International, Inc. (Temecula, CA, USA):

1) Tissue sections were deparaffinized by xylene, ran through ethanol and washed with phosphate buffered saline (PBS).

2) They were then treated with proteinase K (20 μg/ml) and then washed with PBS.

3) Endogenous peroxidase was quenched with 3.0% hydrogen peroxide in PBS for 5 minutes at room temperature and the slides washed with PBS.

4) Equilibration buffer (75 μL/5cm^2) was applied directly on the specimen and incubated for at least 10 minutes at room temperature. Afterwards, a working strength solution of TdT enzyme was applied onto the section (55 μL/5cm^2) and incubated in a humidified chamber at 37 °C for 1 hour.

5) Stop/wash buffer was applied to the sectoins, agitated for 15 seconds, and then incubated for 10 minutes at room temperature.

6) Anti-digoxigenin conjugate was then applied on the section and incubated in a

humidified chamber for 30 minutes at room temperature. The slides were then washed in PBS and developed in peroxidase substrate (75 $\mu L/5cm^2$) to yield a brown product.

7) Specimens were counterstained in 0.5% (w/v) methyl green for 10 minutes at room temperature and dehydrated and mounted.

Apoptosis was observed in the hippocampus and Purkinje cells of the cerebellum under light microscope. Apoptotic cells were calculated in different areas of the hippocampus, the whole hippocampus and Purkinje cell layers. Morphometry was performed by Metamorph version 6.3r6 software (Molecular Devices, Sunnyvale, CA, USA). The apoptotic cells were recorded 5×10^6 μm^2 in whole hippocampus, 1×10^6 μm^2 in subareas of hippocampus, 7×10^5 μm^2 in Purkinje layers.

4.2.7 Antioxidants activity detection

The procedures were performed according to the modified manual from Cayman Chemical Company (Ann Arbor, MI, USA).

4.2.7.1 Tissue homogenate

Three different sets of hippocampus samples were homogenized with a homogenizer in different buffers as follows: 1) 20 mM HEPES buffer, (pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) for SOD; 2) 50 mM potassium phosphate (pH 7.0, containing 1 mM EDTA) for CAT; and 3) 50 mM Tris-HCl (pH 7.5, 5 mM EDTA, and 1 mM DTT) for GPx. The homogenates for SOD assay were centrifuged at 1,500 ×g

for 5 minutes at 4 °C and the supernatant removed for assay, while the homogenates for CAT and GPx assays were centrifuged at 10,000 ×g for 15 minutes at 4 °C.

4.2.7.2 Superoxide dismutase activity

1) SOD standards were prepared as follows: 20 µl of the SOD standard was diluted with 1.98 ml of sample buffer to obtain the SOD stock solution. Seven clean glass test tubes were used and marked as A-G. The SOD stock and sample buffer were added to each tube as below:

Tube	SOD stock (µl)	Sample buffer (µl)	Final SOD activity (U/ml)
A	0	1,000	0
B	20	980	0.025
C	40	960	0.05
D	80	920	0.1
E	120	880	0.15
F	160	840	0.2
G	200	800	0.25

2) SOD standard wells: 200 µl of the diluted radical detector and 10 µl of standard (tubes A-G) per well were added in the designated wells on the plate.

3) Sample wells: 200 µl of the diluted radical detector and 10 µl of sample were added to the wells. Then 20 µl of diluted xanthine oxidase was added to all the wells used. The 96-well plate was placed on a shaker and mixed for a few seconds followed by incubation for 20 minutes at room temperature. The absorbance at 450 nm was read using a Spectra Max250 microplate spectrophotometer (Molecular Devices, Sunnyvale,

CA, USA).

4.2.7.3 Catalase activity

The formaldehyde standards preparation: 10 μl of formaldehyde standard was diluted with 9.99 ml of sample buffer to obtain a 4.25 mM formaldehyde stock solution. Seven clean glass test tubes were marked with A-G. The amount of formaldehyde stock and sample buffer added to each tubes are as follows:

Tube	CAT stock (μl)	Sample buffer (μl)	Final CAT activity (U/ml)
A	0	1,000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

For the formaldehyde standard wells: 100 μl of assay buffer, 30 μl of methanol, and 20 μl of standard (tubes A-G) per well were added to the plate. The positive control (bovine liver CAT) consisting of 100 μl of assay buffer, 30 μl of methanol, and 20 μl of diluted CAT, were added each to two wells. The samples each consisting of 100 μl of assay buffer, 30 μl of methanol, and 20 μl of sample added to the wells. To obtain reproducible results, the amount of CAT added to the wells should result in an activity between 0.25-4 nmol/min/ml. When necessary, samples should be diluted with sample buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight

cut-off of 100,000 to bring the enzymatic activity to this level. Then 20 μl of hydrogen peroxide was added to each of all wells. The samples were incubated on a shaker for 20 minutes at room temperature. Thirty μl of potassium hydroxide was added to each well to terminate the reaction and 30 μl of Purpald (chromogen) to each well was also added. The samples were further incubated for 10 minutes at room temperature on the shaker. Ten μl of potassium periodate was then added to each well and the samples, still on the shaker, were incubated for 5 minutes at room temperature. The absorbance at 540 nm was read by a Spectra Max250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

4.2.7.4 Glutathione peroxidase activity

Background wells were filled with 120 μl of assay buffer and 50 μl of co-substrate mixture. For the positive control wells (bovine erythrocyte GPx), 100 μl of assay buffer, 50 μl of co-substrate mixture, and 20 μl of diluted GPx were added. For the sample wells, 100 μl assay buffer, 50 μl of co-substrate mixture, and 20 μl of sample were added to each of three wells. To obtain reproducible results, the amount of GPx added to the wells should cause an absorbance decrease between 0.02 and 0.135/min. When necessary, samples should be diluted with sample buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to this level. Then 20 μl of cumene hydroperoxide, a GPx substrate, was added to all the wells and then mixed. The absorbance was read at 340 nm with a reader Spectra Max250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), at least 5 times for a sample.

4.2.8 Cell death ELISA

The cell death was detected by ELISA in the hippocampus and Purkinje layer of cerebellum. The procedure was performed according to the modified manual from Roche Diagnostics GmbH (Mannheim, Germany):

One hundred µl of coating solution containing anti-histone antibody was added into each well of the microplate, and the microplate was covered tightly with the adhesive cover foil and incubated for 1 h at 15-25 °C (alternatively overnight at 2-8 °C). The coating solution was rinsed off thoroughly and replaced by 200 µl of incubation buffer into each well. The microplate was tightly covered again and incubated for 30 minutes at 15-25 °C. The wells were rinsed three times with 250-300 µl washing solution per well and the washing solution was discarded. One hundred µl of the sample solution was then added into each well. For determination of the background of the immunoassay, 100 µl per well of incubation buffer was added into two wells, covered and incubated for 90 minutes at 15-25 °C. The wells were rinsed three times with 250-300 µl washing solution per well and the washing solution was discarded. One hundred µl of conjugate solution (anti-DNA-peroxidase (POD)) was added into each well, except that at the blank position, and then the microplate was covered and incubated for 90 minutes at 15-25 °C. The wells were washed again and 100 µl of substrate solution (2, 2'-azino-di-[3-ethylbenzthiazoline sulfonate], ABTS) was added into each well and was incubated on a plate shaker at 250 rpm until the color developed was intense enough for a photometric analysis. The wells were measured at 405 nm by a microplate reader Spectra Max250

microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The background value was then subtracted from the value of each sample.

4.2.9 Statistical analysis

All data were presented as mean ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by a post hoc comparison test using Fisher's LSD test or independent t-test to confirm the significant difference between groups. $P \leq 0.05$ was considered statistically significant. All statistical procedures were performed with the software SPSS16.0 (SPSS Inc, Chicago, IL, USA).

4.3 Results

4.3.1 Body weight

The time-course of the change in body-weights after brain ischemia in SHR and WKY rats were analyzed at day 15, 30 and 60 after the occlusions of the common carotid artery (Figure 4.2); the body weight at day 0 was used as the control. We found that at day 0 there were no differences between the body weights of the four groups (SHR and WKY). At day 15 after the operation, the body weight decreased in all 4 groups compared with the weights at day 0; however, the decreases were not statistically significant. At day 60, the body weight increased as compared to the weights at day 0 and 15, and these increases were statistically significant. In particular, in rats of the SHR-ischemia group, the weight was significantly higher than the weight at day 30.

There were no significant differences in body weights between groups at different time points.

4.3.2 Brain weight

After the sham or ischemic operation 6 rats of each group of SHR or WKY (sham and ischemia) were sacrificed and the brains were removed and weighed individually at day 0, 15, 30 and 60. As shown in Figure 4.3, at day 0 the brain weights of SHR-sham were lower than those of WKY-sham rats ($p < 0.05$). The decline was seen till day 60; the brain weight of WKY-ischemia rats at day 60 was significantly lower when compared to WKY-sham rats ($p < 0.05$).

4.3.3 Brain weight:body weight ratio

After sham or ischemic operation of SHR and WKY rats, the brain weight to body weight ratios of all rats at different times were evaluated. As shown in Figure 4.4, SHR-sham showed lower brain-body weight ratios than the corresponding age-matched WKY-sham at all time points ($p < 0.05$). At day 15 after the operation, SHR-ischemic rats gained higher brain-body weight ratio than the SHR-sham rats ($p < 0.05$); however, the brain-body weight ratio of WKY-ischemic rats displayed lower changes than WKY-sham rats at day 60 ($p < 0.05$).

4.3.4 Nissl staining

Under the light microscope, the CA1 area (the most vulnerable area in brain ischemia) was studied. The following findings were observed. In the sham animals, pyramidal cells in the CA1 area were aligned in 3-4 layers, with large and round nuclei and clear nucleoli. Very occasional, a constricted (triangular at times) nucleus was observed in WKY-sham (Figure 4.5 A) and in SHR-sham rats (Figure 4.5 B) day 15 after the operation. For the WKY strains, both the WKY ischemic and sham rats showed no apparent changes (Figure 4.5 C to E) until day 60 after the operation. At this time, CA1 pyramidal cells were disoriented, and many showed nuclear pyknosis. The nuclear membrane and nucleoli were fuzzy and the nucleus shrunken (Figure 4.5 G). On the other hand, in the SHR, particularly in the SHR-ischemia rats, the CA1 area showed large area of nuclear pyknosis at day 15 after the operation (Figure 4.5 D). By 30-60 days, however, pyknosis was minimal, but the pyramidal cells were not tightly grouped together and there were pyknotic nuclei (Figure 4.5 F to H). In the Purkinje cell layer of the cerebellum, histologically, not much change was observed between the different groups of WKY and SHR (Figure 4.6).

4.3.5 Apoptosis of hippocampus and cerebellar Purkinje cells after brain ischemia

The TUNEL experiment showed that the nucleus of apoptotic cells was stained brown and the cell body shrunk to round, triangle in shape. Some apoptotic cells were broken into several apoptosis bodies. Positive apoptotic cells were counted. At different time points, apoptotic cells could be seen in different areas of the hippocampus. Results from both WKY and SHR sham rats showed no significantly change in apoptotic numbers of hippocampal cells at all time points (Figure 4.7 A and B). After ischemia operation,

apoptotic cell number of the hippocampus gradually increased from day 15 to day 60 in WKY-ischemia rats compared with that of the respective WKY-sham rats; the difference was especially noticeable at day 60 (9.18 ± 2.04 vs 1.71 ± 0.46 respectively for WKY-ischemia and WKY-sham rats, $p < 0.05$) (Figure 4.7 C, E and G). However, in SHR-ischemia rats the apoptotic cell number significantly increased at day 15 compared with that of the corresponding sham rats (17.22 ± 5.23 vs 0.74 ± 0.46 respectively for SHR-ischemia and SHR-sham rats, $p < 0.05$) (Figure 4.7 D), and then declined at day 30 and 60 (Figure 4.7 F and H, and Figure 4.8). The apoptotic cells were mainly found in the CA1 region of the hippocampus. In the cerebellum, we also found apoptotic cells in the Purkinje cell layer with variable changes over time points (Figure 4.9 and 4.10). In general, there was an increase of apoptosis of these cells 60 days after the lesion in both the sham and experimental SHR strain. However, the differences between the ischemia and sham groups were not statistically significant in the 3 time points after the operation.

4.3.6 Antioxidants activity in hippocampus

4.3.6.1 Superoxide dismutase (SOD) activity assay

SOD activity (mean ± SD) in the hippocampus was significantly lower in SHR-sham rats than in WKY-sham rats at day 15 (15.63 ± 10.65 vs 131.05 ± 21.48 respectively, $p < 0.05$), day 30 (327.67 ± 49.91 vs 404.67 ± 53.77 respectively, $p < 0.05$) and day 60 (6.40 ± 1.18 vs 10.77 ± 3.15 respectively, $p < 0.05$) after the operation (Figure 4.11). After the arterial occlusion, SOD activities in SHR-ischemia rats increased significantly as compared to the SHR-sham rats at day 15 (47.45 ± 8.80 vs 15.63 ± 10.65 respectively,

$p < 0.05$), day 30 (551.83 ± 44.90 vs 327.67 ± 49.91 respectively, $p < 0.05$) and day 60 (16.02 ± 3.71 vs 6.40 ± 1.18 respectively, $p < 0.05$) (Figure 4.11). On the other hand, SOD activities in WKY-ischemia rats decreased significantly as compared to WKY-sham rats by day 15 (60.62 ± 8.60 vs 131.05 ± 21.48 respectively, $p < 0.05$) and day 30 (329.83 ± 40.67 vs 404.67 ± 53.77 respectively, $P < 0.05$), but increased significantly at day 60 (15.65 ± 3.45 vs 10.77 ± 3.15 respectively, $p < 0.05$) (Figure 4.11). The highest increase was at day 30 when SOD of SHR-ischemia is about 500 U/ml and the increases of SOD activities in the SHR were in general earlier than those in the WKY (Figure 4.11).

4.3.6.2 Catalase (CAT) activity assay

CAT activities in the hippocampus of SHR-sham rats were similar to those of WKY-sham rats at day 15, 30 and 60, indicating that SHR possibly had similar free radical scavenging capability by CAT in the hippocampus as WKY rats (Figure 4.12). All the sham and ischemia groups of SHR and WKY had the highest levels of CAT activity (about 500 mmol/min/ml) at day 15 after the operation and this activity decreased to 20-40 mmol/min/ml by day 30 and to 7-9 mmol/min/ml at day 60. This showed that the effect of the stress of the operation on catalase production and activity was most pronounced on day 15 after the surgery. The results also confirmed that, as far as this enzyme was concerned, there was significant increase in the SHR-ischemia group as compared to the SHR-sham group at day 15 (550.30 ± 33.74 vs 483.13 ± 30.80 respectively, $p < 0.05$) and day 30 (38.62 ± 4.65 vs 17.15 ± 1.35 respectively, $p < 0.05$) after operation. The ischemia led to an increase of CAT activity, which seemed to return

to normal by day 60 since the SHR-ischemia group showed no significant difference as compared to the SHR-sham group (Figure 4.12). For WKY, the ischemia groups did not cause any increase in CAT activities as compared to the sham groups at all three time points. Consequently, there were no significant differences between the WKY-sham, WKY-ischemia and SHR-sham groups (Figure 4.12). This strongly suggested that only the hypertensive strain had significant aggravation after ischemia and thus significantly more antioxidant activity was induced.

4.3.6.3 Glutathione peroxidase (GPx) activity assay

GPx activities in the hippocampus in both SHR-sham and WKY-sham were similar at the 3 time points, indicating that SHR possibly had similar ability as WKY rats in free radical scavenging by GPx in the hippocampus (Figure 4.13). After the operation, GPx activity in SHR did not change significantly at the 3 time points (from 160 mmol/min/ml at day 15 postoperative to 60 mmol/min/ml at day 60 postoperative). WKY, on the other hand, did not follow the same trend. Indeed, WKY-ischemia group at day 15 showed significantly lower GPx activity as compared to the WKY-sham group (61.68 $\pm$ 7.12 vs 146.18 $\pm$ 15.04 respectively, $p < 0.05$) (Figure 4.13). At day 30 after the operation, GPx activity was still significantly lower in WKY-ischemia group as compared to WKY-sham group (43.57 $\pm$ 7.32 vs 67.20 $\pm$ 15.46 respectively, $p < 0.05$) (Figure 4.13). However, the activity in WKY-ischemia group increased to a level at day 60 that show no significant difference from the WKY-sham group (Figure 4.13). This showed that the WKY was slow in reacting to the ischemia, and the injury was less than that of SHR because of the absence of hypertension. Alternatively, there were other differences

between the two species that might lead to the different changes in GPx activity in response to ischemia.

4.3.7 Cell death in hippocampus and cerebellum

In the hippocampus, both the SHR- and WKY-ischemia rats showed significantly higher levels of cell death than those of the corresponding WKY- and SHR-sham rats ($p < 0.05$) (Figure 4.14). In the cerebellum, both the WKY- and SHR-ischemia rats also showed higher levels of cell death than the WKY- and SHR-sham rats, but the differences were not significant (Figure 4.15). There was also no significant difference between the four groups.

4.4 Discussion

It is well known that the rats have a very similar pattern of brain blood vessels as human, In both species, the cerebral supply consists of the carotid artery system and vertebral-basilar artery system (Lee, 1995). When both the left and right common carotid arteries were blocked, the affected brain areas would derive blood supply from the vertebral-basilar system through the circle of Willis. The blood supply of the vertebral artery system would show a corresponding reduction due to this compensatory role (Sarti, et al., 2002). The blood in the hippocampus and cerebellum was supplied by the carotid and the vertebral-basilar arterial systems respectively. Previous research have confirmed that the hippocampus (particularly its CA1 region) was one of the brain regions most sensitive to ischemia. The hippocampus has a distinct laminar structure and the synaptic

connections have been precisely mapped to allow the exact cell type (e.g. pyramidal cells) or layers to be studied (Farkas, et al., 2007). Therefore, we chose the hippocampus and cerebellum, which are most sensitive to ischemia, to study the effects of ischemia or the compensatory blood supply reduction on the protective mechanisms and survivability of neurons.

Our experimental results showed that there was no significant difference between SHR and aged-matched WKY rat in body weight after the cerebral ischemia surgery and sham surgery. On the whole, the body weights of each group after surgery at day 15 showed a downward trend, while at day 30 and at day 60, the body weight exhibited a gradual increase trend. However, at the same time point there was no significant difference in body weight between the ischemia and sham rats, showing that cerebral ischemia itself did not affect the body weight. Similar to the other major surgeries, certain muscles (e.g. the sternohyoid and the sternomastoid muscles) showed slight damages in the ventral cervical region after the bilateral ligation of the carotid artery. Other clinical symptoms such as discomfort during movement of the head, mastication and swallowing perhaps caused this initial loss of body weight (Farkas, et al., 2007). The increase of weight 30-60 days after surgery as a whole showed that the animals were recovering and the feeding habit was returning to normal.

This study showed that the brain weights and brain weight:body weight ratios of WKY-sham rats were higher than SHR-sham rat at three time points. This is consistent with results from the a previous study (Nelson, et al., 1993), both showing a possible difference between the two species. Our results also indicated that the brain weight and

brain weight to body weight ratio had significant declined in WKY-ischemia rats compared to WKY-sham rats. This was possibly due to encephalatrophy after the long-time ischemia (Berne, et al., 2009). On the other hand, the significant increases in brain weight to body weight ratio in SHR-ischemia rats compared with the SHR-sham rats might be due to brain edema caused by ischemia (Irisawa, et al., 2008). Finally, mild increase of brain weight may be also due to gliosis (Becker and Takashima, 1985).

It is well known that because of the disturbance to energy metabolism by cerebral ischemia (Zhang, et al., 2001), brain cells could produce a large amount of free radicals, leading to oxidation of unsaturated fatty acid in the membranes. Free radicals and malondialdehyde (MDA) produced during lipoperoxidation would damage the structure of cell membrane and biological macromolecules inside the cells, leading to possible cell death (Huie and Padmaja, 1993). There are two types of cell death, necrosis and apoptosis Generally, necrosis occurs in the case of severe damage, and less severe or mild damage leads to apoptosis (Srinivasan, et al., 1996). Generally, it is believed that the ischemia induced by the bilateral ligation of the common carotid artery is relatively mild in its chronic phase. In this lesion there is a delayed neuronal death in the hippocampus (Srinivasan, et al., 1996).

At present, the Wister rats, Sprague-Dawley (SD) rat and WKY rat were the most widely used animals to investigate the effects of chronic brain ischemia caused by the bilateral ligation of the carotid artery; however the pathological data and the extent of injured areas in these animals were inconsistent (Ulrich, et al., 1998; Otori, et al., 2003; Choy, et al., 2006). The pathology suggested that different animals had different

tolerance to ischemia and this may be due to difference in strains. Even for the same species and strains, there may be variations between the different breeds or batches. Another important factor is the use of different anesthetic agents, which do not all have the same neural mechanism (Miura, et al., 1998; Nellgard, et al., 2000).

In the present study, Nissl staining showed that in the sham WKY rats and SHR at day 15, 30 and 60 after operation, pyramidal cells in the CA1 area were aligned well and had a large and round nucleius and clear nucleoli. Very occasional, a constricted nucleus was visible. After an ischemia for 60 days, CA1 pyramidal cells of the WKY rar had become disoriented, and many showed nuclei pyknosis. The nuclear membrane and nucleoli were fuzzy and the nuclei shrunk. On the other hand, for the SHR, particularly in the SHR ischemia rats, the CA1 area showed large area of nuclear pyknosis at day 15 after the operation. By 30 and 60 days, however, pyknosis was minimal, but the pyramidal cells were not tightly aligned together and there were pyknotic nuclei.

The present TUNEL results also showed the same pattern. In the CA1 region of the hippocampus of WKY-ischemia rats, the number of apoptotic cells gradually increased from 15 to 60 days after ischemia, but in the SHR-ischemia rats, the number of apoptotic cells in this region at day 15 was more than those at day 30 and day 60. This suggested that in WKY rats the ischemic damage in the hippocampus CA1 region was a step-by-step process and the damage extent was relatively mild as compared to that in SHR. In the SHR, there were probably more severe damages in hippocampus CA1 region at day 15, followed by a slow recovery. ELISA also indicated that the total hippocampal cell death was significantly increased 15 days after ischemia. Therefore, in the subsequent

experiments, 15 days after the occlusion surgery was regarded as the best time point to investigate the induced cell death.

In the cerebellum, the TUNEL staining results showed that there was an increase of apoptosis in the Purkinje cells 60 days after the operation in both the SHR sham and ischemia groups. The difference between the two groups was however insignificant at the three time points after the operation. Both the Nissl staining and ELISA results showed that there were no significant differences in cell death at different time points amongst the groups. One possible reason is that the cerebellum was supplied by the vertebral-basilar system and the occlusion of the common carotid did not affect this area as much as the hippocampus. Therefore, in the following biochemical analyses, we would concentrate on the hippocampus, and not the cerebellum.

Under normal physiological conditions, the aerobic metabolism of the body can produce a small amount of free radicals, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and so on (Inal, et al., 2001). If these free radicals could not be removed promptly, the proteins, lipids and nucleic acids will be oxidized and then our tissues will be damaged. Under normal circumstances, there is a dynamic equilibrium between the production and the removal of free radicals, but in ischemia, especially during reperfusion, the free radicals generated were too much to be removed by the endogenous antioxidation system in time (Liu, et al., 2001). Studies have shown that free radicals-related metabolic disorders caused by chronic hypoperfusion of the brain may be an important factor of brain damage in arterial occlusion (Tanaka, et al., 2002).

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are the major enzymes that can remove different kinds of free radicals (Inal, et al., 2001). Among these enzymes, SOD is the only enzyme which can remove superoxide anions (O_2^-) in our body, so it is the key enzyme of the antioxidation enzyme system (Guemouri, et al., 1991). SOD can catalyze free radical (O_2^-) to O_2 and H_2O_2 by disproportionation, which in turn is cleared by the catalase, GPx and so on (Figure 4.16). So the level of SOD activity in our body can indirectly indicate the degree of cell damage and the ability to eliminate free radicals (Rudnicki, et al., 2007). Glutathione peroxidase (GPx) catalyzes the oxidation of glutathione (GSH) with the reduction of hydrogen peroxide, thus helping protect the cell membrane and the essential molecules (Ye, et al., 2000) (Figure 4.16).

Our experimental results showed that under normal circumstances, SOD activity in the hippocampus of SHR rats was lower than that of WKY rats, which indicated there was production of free radicals caused by hypertension, which could chronically consume SOD, finally result in low level of SOD. After ischemia for 15, 30 and 60 days, SOD activity exhibited an obvious increase in the hippocampus of SHR rats when compared with that of the sham group. The highest increase is at day 30. The increase would protect the body against oxidative damage. In contrast, the SOD activity of the ischemic WKY rats decreased dramatically, till its recovery at day 60. This suggested that WKY also reacted to injury, but at a slower rate, probably because the extent of lesion was not as great as the SHR, but also perhaps was related to species difference. The earlier increase in SOD of the SHR than the WKY reflected the more severe injury in the SHR strain.

Our results showed that CAT activities in the hippocampus of the SHR-sham rats and WKY-sham rats were similar, indicating that SHR possibly had similar capability of CAT-related free radical scavenging in the hippocampus as WKY. All groups of SHR and WKY had the highest levels of CAT activity at day 15 after the operation. This showed that the stress due to the operation had an impact on CAT production. In terms of CAT activity, SHR rats had higher levels than the corresponding sham rat groups, but there was no difference between the WKY ischemia and sham rats. This suggested that the hypertensive rats suffered a more severe aggravation and therefore needed to maintain a higher level of antioxidation activity against the adverse effects of ischemia.

GPx activities in the hippocampus in both the SHR-sham and WKY-sham had a similar trend as CAT, and was highest at day 15 postoperation. This showed that SHR possibly had similar ability to GPx in free radical scavenging in hippocampus as WKY rats. Furthermore, the GPx activities of WKY-ischemia groups were low at the 15 and 30 days after the operation, but increased by day 60. This showed again that the WKY was slow in reacting, again possibly became the ischemia was not as injurious as that in the SHR, or there were some other differences between the two strains.

4.5 Conclusion

Chronic brain ischemia combined with hypertension could increase the damage of ischemia and hypoxia in the rat hippocampus. This was evidenced by the increased activities of the antioxidation enzymes including SOD, CAT and GPx. In the present study, chronic ischemia was induced by interrupting the carotids or their branches, so the

cortical areas were much affected. Apart from a general increase in antioxidation activities, cell death in general (regardless of apoptosis or necrosis) and apoptosis as reflected by TUNEL were all increased. The increase was particularly significant at day 15 and 30 after the ischemia. Although blood from the vertebrobasilar system would supply the cortical area after carotid lesion, the compensation was apparently not sufficient. This aligned well with the clinical findings that deficits were most evident 15 days after ischemic stroke (Lee, et al., 2008). The cerebellum, used here to represent a region belonging to the vertebrobasilar distribution, was also affected but to a lesser extent.

Table 4.1 Number of rats used in different experiments; a total of 60 WKY rats and 60 SHR were used

	WKY-sham			WKY-ischemia			SHR-sham			SHR-ischemia			Total
	15 d*	30 d*	60 d*	15 d	30 d	60 d	15 d	30 d	60 d	15 d	30 d	60 d	
Morphology including Nissl and TUNEL staining	4	4	4	4	4	4	4	4	4	4	4	4	48
Antioxidation activity, cell death ELISA and brain weight	6	6	6	6	6	6	6	6	6	6	6	6	72
Total	10	10	10	10	10	10	10	10	10	10	10	10	120

*d: days after operation

Table 4.2 Tissue process and embedding

Chemical	Concentration (%)	Time (h)	Temperature (°C)	Vacuum
ethanol	70	3	ambient	
ethanol	80	3	ambient	
ethanol	95	3	ambient	
ethanol	100	2	ambient	
ethanol	100	2	ambient	
ethanol	100	2	ambient	
xylene		2	ambient	
xylene		2	ambient	
wax		2	60	vacuum
wax		2	60	vacuum
wax		2	60	vacuum
wax		2	60	vacuum

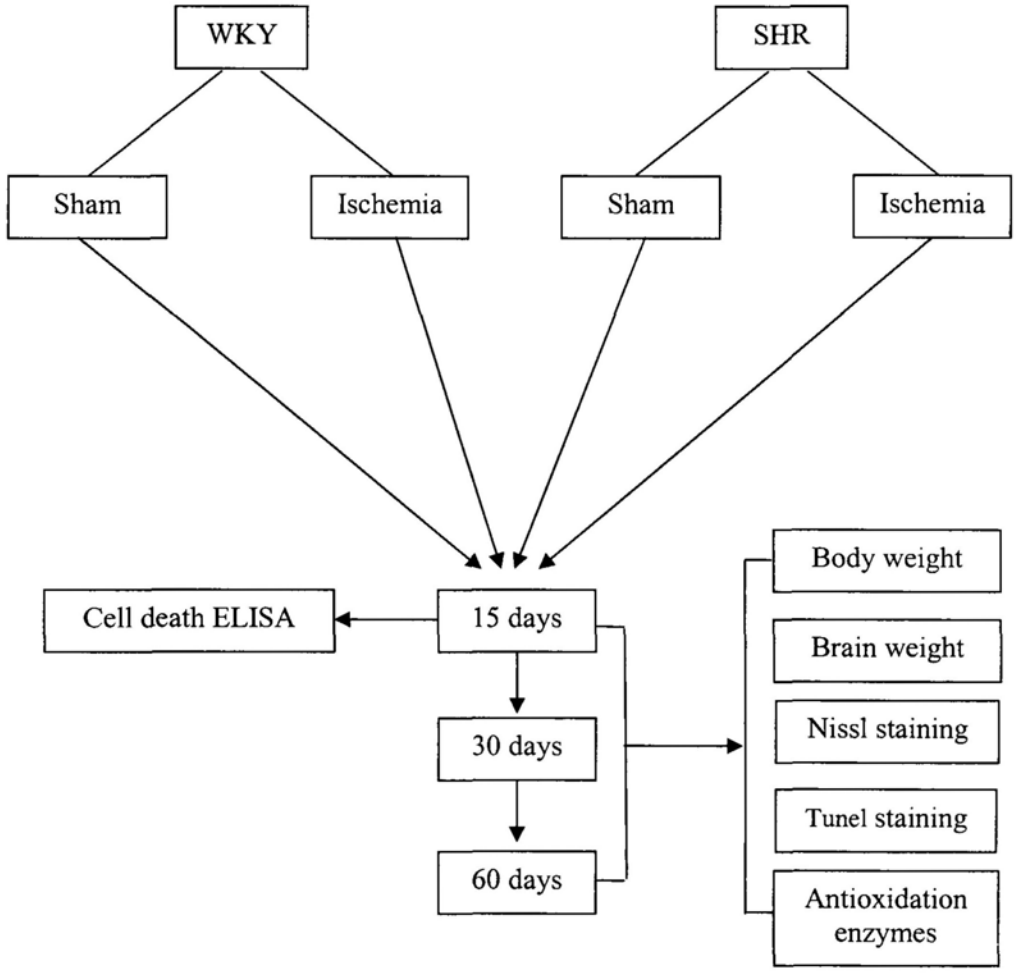


Figure 4.1 Flowchart of different experimental procedures.

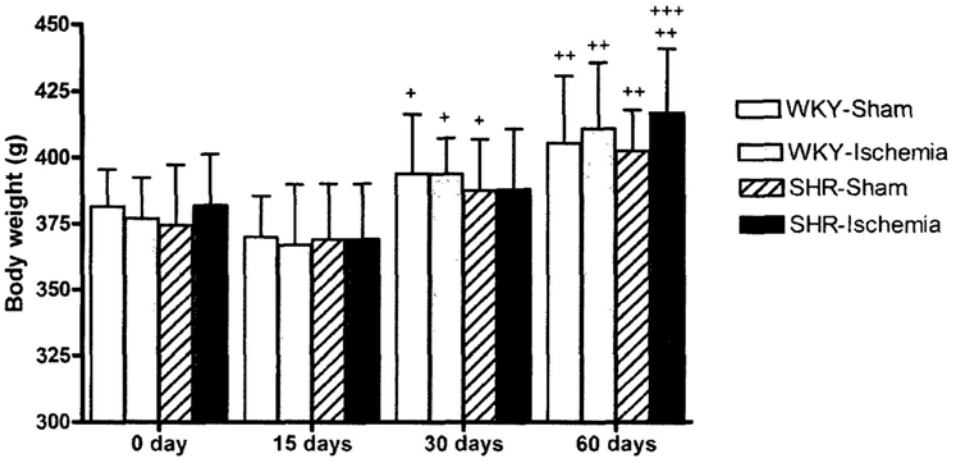


Figure 4.2 The variations of body-weights with time after the ischemia operation in SHR and WKY rats. The body weights of the SHR and WKY rats were weighed after sham or ischemic operation at different times (0, 15, 30 and 60 days). Data were the means ± SD. Significance of test for difference: +: $p < 0.05$, in comparison with rats at 15 days; ++: $p < 0.05$, in comparison with rats at 15 days and 0 day; +++: $p < 0.05$, in comparison with rats at 30 days.

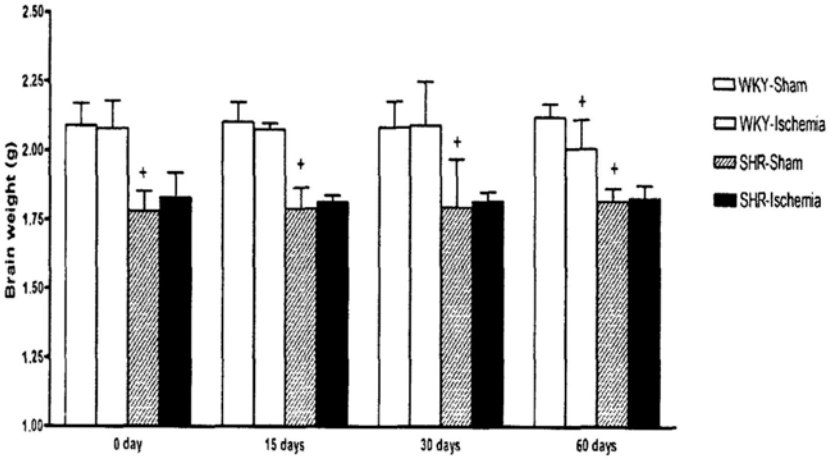


Figure 4.3 The variations of brain-weights with time after operation in SHR and WKY rats. The brains of the SHR and WKY rats were removed and weighed after sham or operation of brain ischemia at different time (0, 15, 30 and 60 days). Data were means ± SD. Significance of test for difference from WKY-sham group is $p<0.05$ at +.

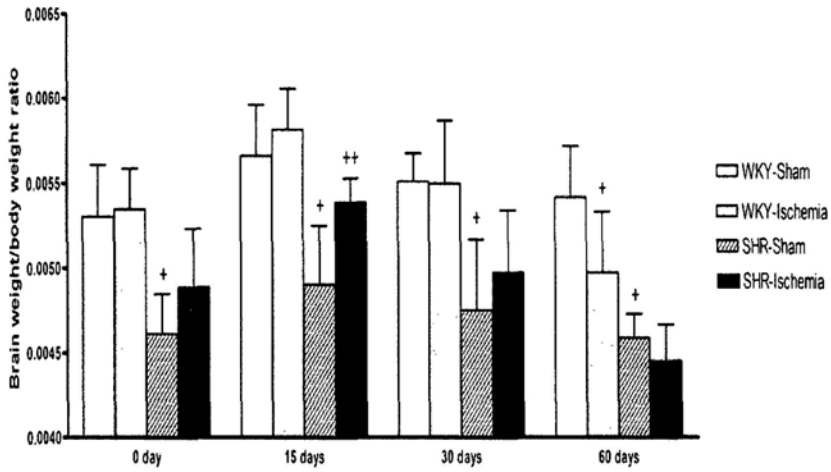


Figure 4.4 The variations of brain-weight:body weight ratios with time-course after ischemia operation in SHR and WKY rats. At different time after sham or operation (0, 15, 30 and 60 days) the body weight of each rat was weighed; then six of the SHR and WKY rats in each group were anesthetized by chloral hydrate and killed. The brains were removed and weighed. The brain weight:body weight ratio was calculated and statistically compared between groups. Data were mean ± SD. Sign + indicates significant difference with $p < 0.05$ from WKY-Sham rat; ++ indicates significant difference with $p < 0.05$ from SHR-sham rat.

Figure 4.5 Nissl staining of CA1 of hippocampus in SHR and WKY rats. **A** and **C** represented brains of WKY-sham and WKY-ischemia respectively 15 days after operation. **E** and **G** represented brains of WKY-ischemia 30 and 60 days respectively after operation. **B** and **D** represented brains of SHR-sham and SHR-ischemia respectively 15 days after operation. **F** and **H** represented SHR-ischemia brains 30 and 60 days respectively after operation. Black arrows indicated condensed neurons while red arrows indicated neurons with small size due to the shrinkage. Scale bar = 50 μm.

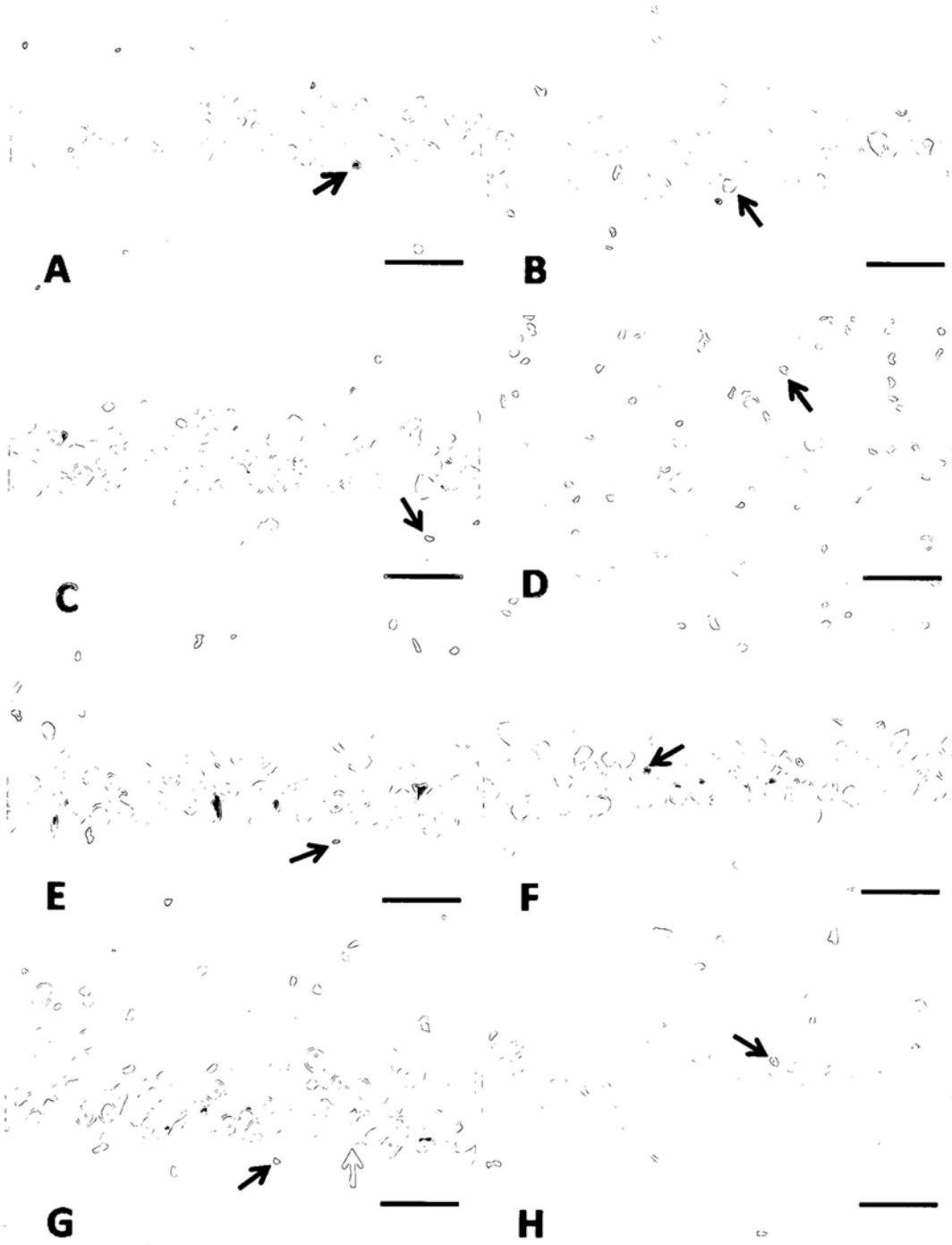

A
B
C
D
E
F
G
H

Figure 4.6 Nissl staining of Purkinje cell layer of the cerebellum in SHR. **A**, **C** and **E** respectively represented Purkinje cell layer of SHR at 15, 30 and 60 days after sham operation. **B**, **D** and **F** respectively represented Purkinje cell layer of SHR at 15, 30 and 60 days after ischemic operation. Comparison between the sham-operated group and ischemic group at 15 days, 30 days and 60 days showed no obvious histological changes in Purkinje cells. Scale bar = 50 μm.

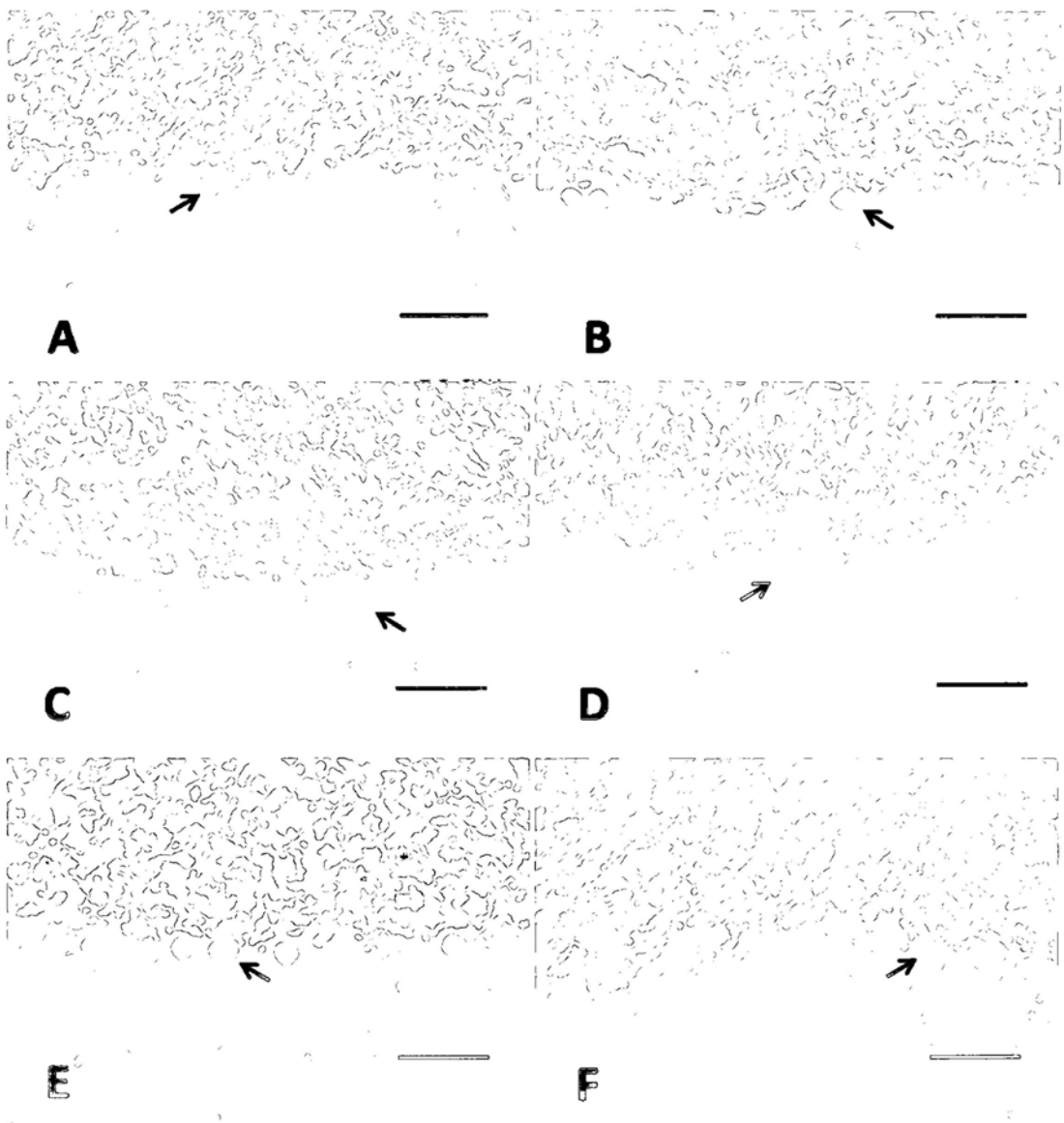

A
B
C
D
E
F

Figure 4.7 TUNEL staining of CA1 of hippocampus in SHR and WKY rats. **A** and **C** represented brains of WKY-sham and WKY-ischemia 15 days respectively after operation. **E** and **G** represented WKY-ischemia brains 30 and 60 days respectively after operation. **B** and **D** represented brains of SHR-sham and SHR-ischemia 15 days respectively after operation. **F** and **H** represented SHR-ischemia brains 30 and 60 days respectively after operation. Black arrows pointed to the apoptotic cells. Scale bar = 50 μm.


A
B
C
D
E
F
G.
H

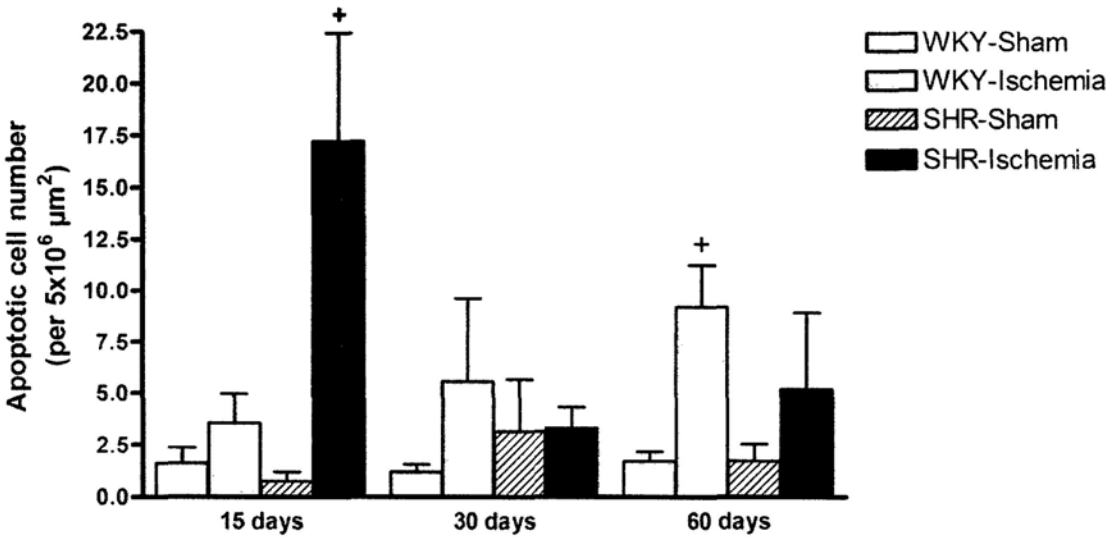
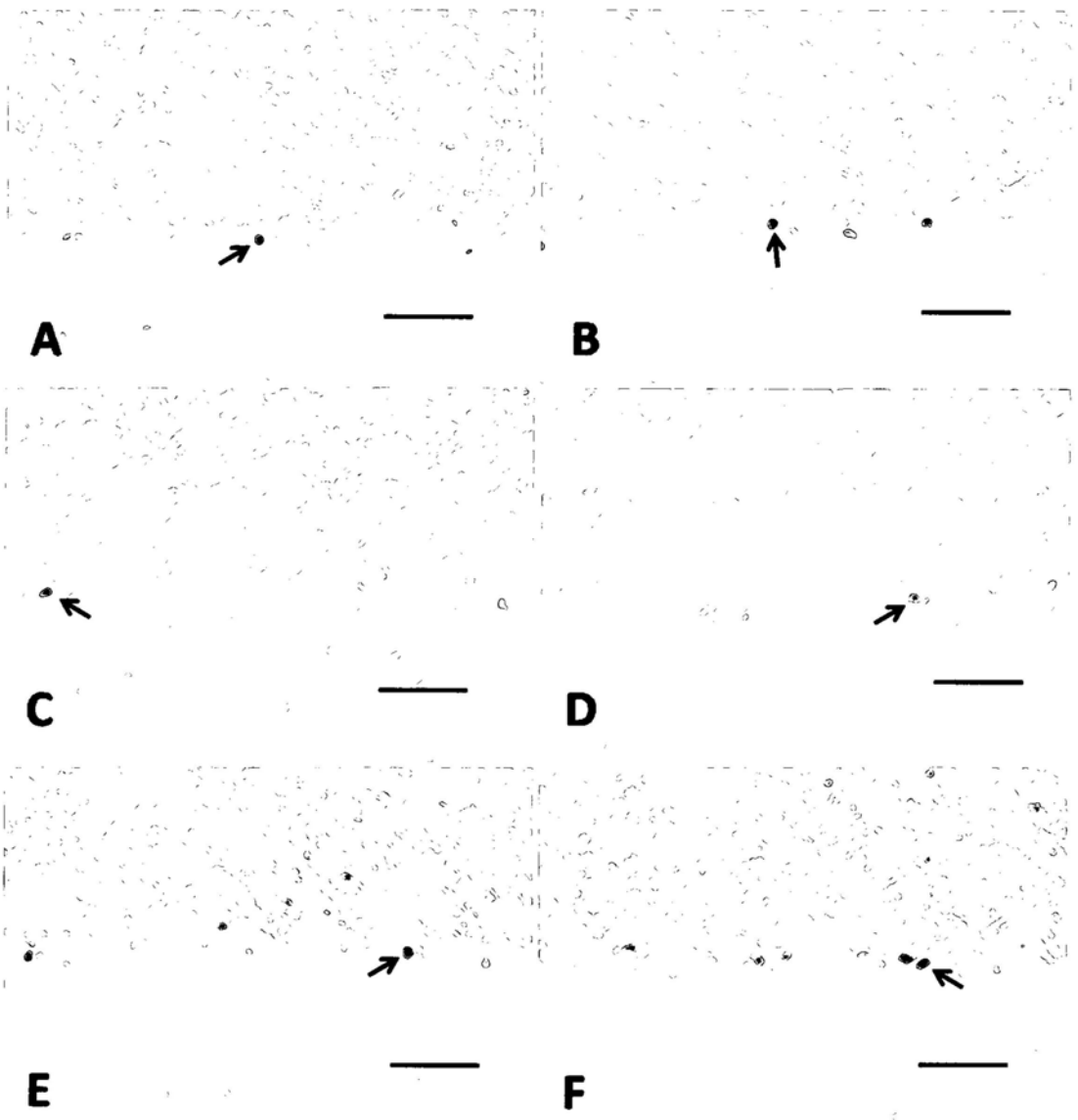


Figure 4.8 Numbers of cells stained positively with TUNEL for apoptosis, in hippocampus of SHR and WKY rats after sham or ischemia operation at 15, 30, 60 days. At 15, 30, 60 days after ischemia operation, the rats were anesthetized by chloral hydrate and sacrificed, and then the brain was removed and fixed with 4% paraformaldehyde. The samples were sectioned and cell death (apoptosis) of hippocampal cells was detected by TUNEL. Data were presented as mean ± SD. Sign + indicates significant difference with $p < 0.05$ from the respective sham rats.


A
B
C
D
E
F

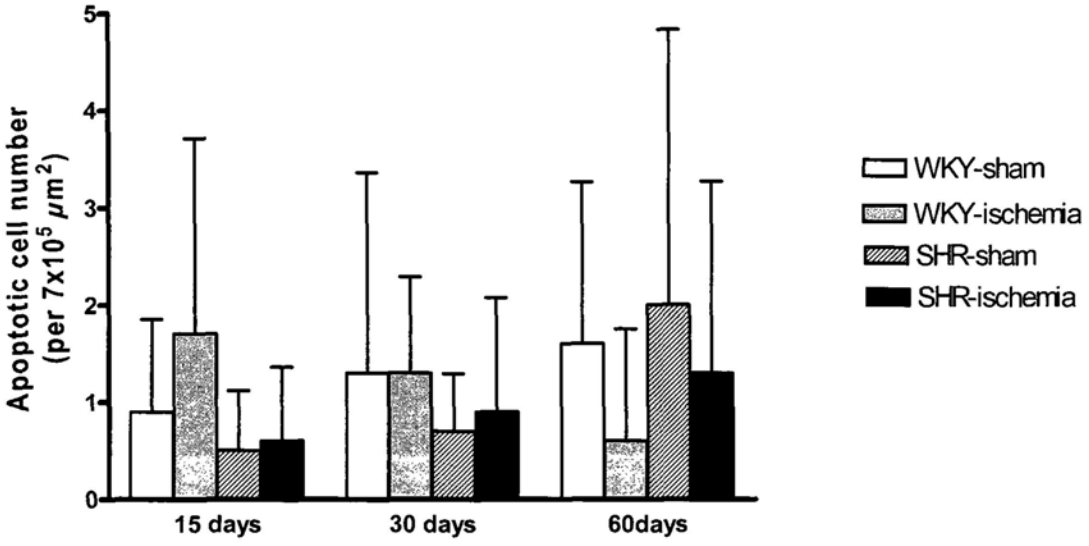
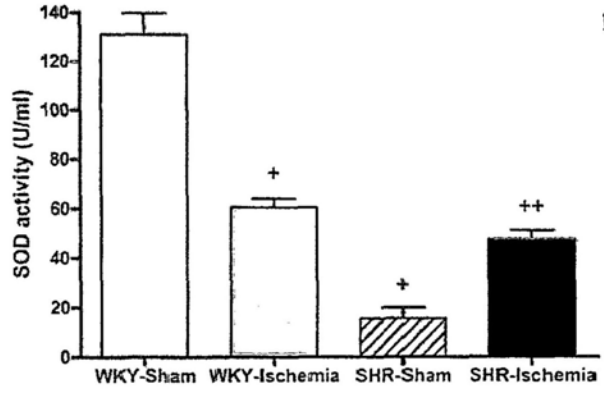
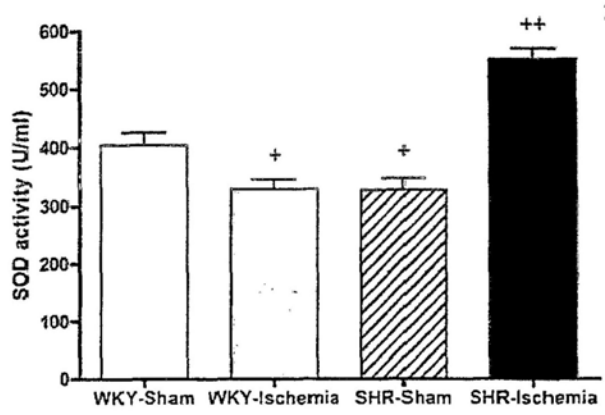


Figure 4.10 Numbers of Purkinje cells stained positively with TUNEL for apoptosis, in SHR and WKY rats after sham or ischemia operation at 15, 30, 60 days. At 15, 30, 60 days after operation, the rats were anesthetized by chloral hydrate and sacrificed, and the brain was removed and fixed with 4% paraformaldehyde. Then the samples were sectioned and cell death (apoptosis) of cerebellar Purkinje cells were detected by TUNEL method. Data were mean ± SD.

Figure 4.11 SOD activity of hippocampus in SHR and WKY rats after sham or ischemia operation at 15, 30, 60 days. SHR-sham rats had significantly lower SOD activities than WKY-sham rats ($p < 0.05$). After ischemia operation, SOD activities decreased significantly at 15 and 30 days in WKY-ischemia group compared with those in WKY-sham groups ($p < 0.05$). When the ischemia continued to a longer period of 60 days, the SOD activity of WKY-ischemia rats increased significantly as compared to the WKY-sham rats ($p < 0.05$). In SHR rats, SHR-ischemia groups showed significant increases in the SOD activities as compared to SHR-sham groups at all time points after operation ($p < 0.05$). Sign + indicates significant difference with $p < 0.05$ from respective WKY-sham rats; ++ indicates significant difference with $p < 0.01$ from respective SHR-sham rats.


15d
SOD activity (U/ml)
140
120
100
80
60
40
20
0
+
+
++
WKY-Sham
WKY-Ischemia
SHR-Sham
SHR-Ischemia


30d
SOD activity (U/ml)
600
500
400
300
200
100
0
+
+
++
WKY-Sham
WKY-Ischemia
SHR-Sham
SHR-Ischemia

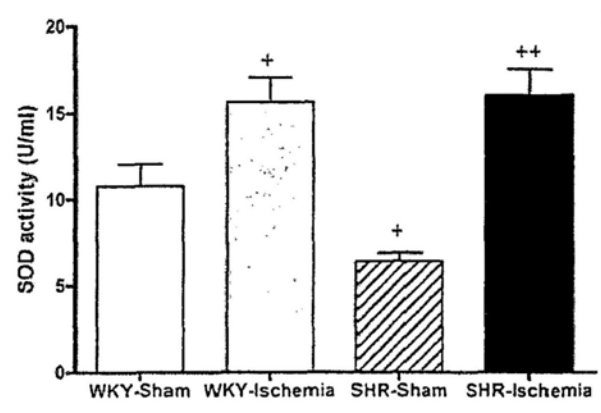

60d
SOD activity (U/ml)
20
15
10
5
0
+
+
++
WKY-Sham
WKY-Ischemia
SHR-Sham
SHR-Ischemia

Figure 4.12 CAT activity of hippocampus in SHR and WKY rats at 15, 30, 60 days after sham or ischemia operation. The catalase activities in SHR-ischemia groups were significantly higher than those in the respective SHR-sham groups at 15 days and 30 days ($p < 0.05$). But at 60 days, there was no difference between the two group. Sign + indicates significant difference with $p < 0.05$ from SHR-sham rats.

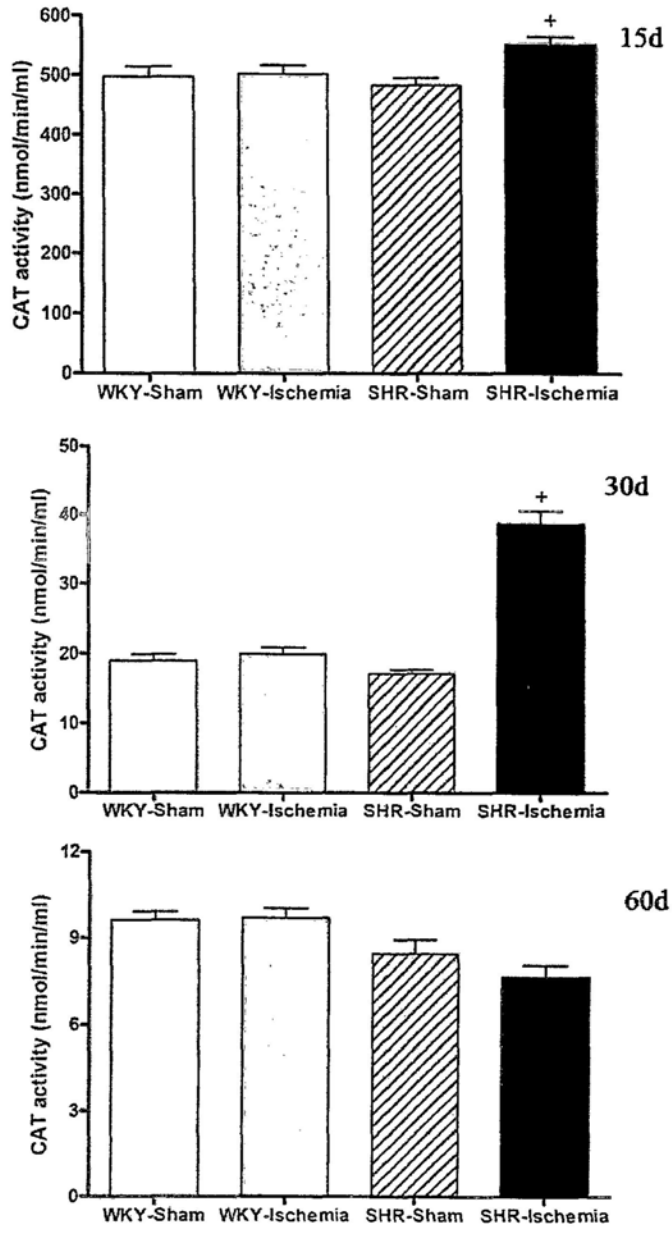
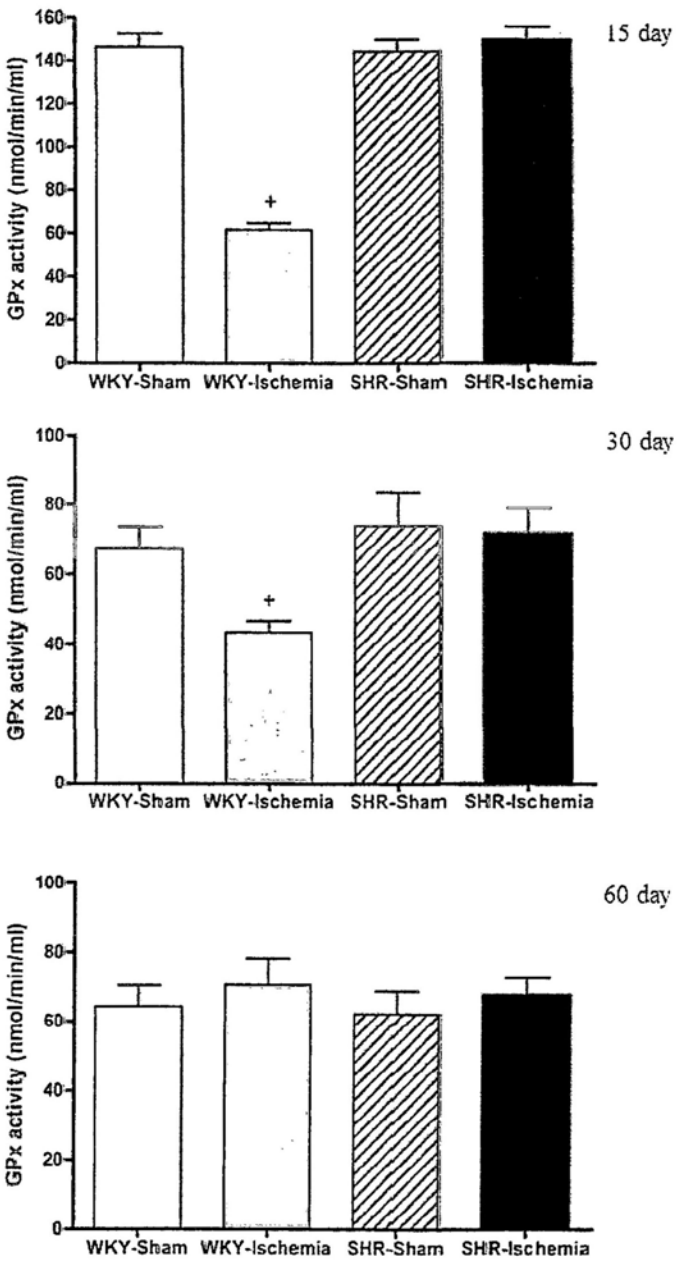

15d
CAT activity (nmol/min/ml)
600
500
400
300
200
100
0
+
WKY-Sham
WKY-Ischemia
SHR-Sham
SHR-Ischemia
30d
CAT activity (nmol/min/ml)
50
40
30
20
10
0
+
WKY-Sham
WKY-Ischemia
SHR-Sham
SHR-Ischemia
60d
CAT activity (nmol/min/ml)
12
9
6
3
0
WKY-Sham
WKY-Ischemia
SHR-Sham
SHR-Ischemia

Figure 4.13 GPx activity of hippocampus in SHR and WKY rats at 15, 30, 60 days after sham or ischemia operation. The GPx activities in WKY-ischemia groups were significantly lower than those in WKY-sham groups at 15 days and 30 days. However, there was no significant difference between these two groups at 60 days. There were no significant differences in GPx activities between SHR-sham groups and SHR-ischemia groups at the three time points. Sign + indicates significant difference with $p < 0.05$ from respective WKY-sham rats.


15 day
GPx activity (nmol/min/ml)
160
140
120
100
80
60
40
20
0
+
WKY-Sham
WKY-Ischemia
SHR-Sham
SHR-Ischemia
30 day
GPx activity (nmol/min/ml)
100
80
60
40
20
0
+
WKY-Sham
WKY-Ischemia
SHR-Sham
SHR-Ischemia
60 day
GPx activity (nmol/min/ml)
100
80
60
40
20
0
WKY-Sham
WKY-Ischemia
SHR-Sham
SHR-Ischemia

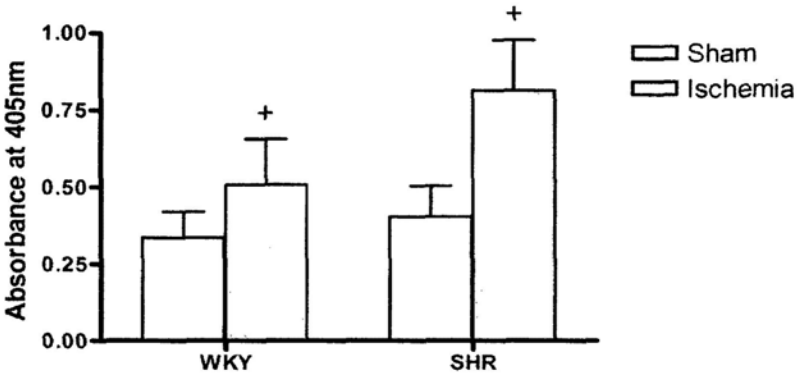


Figure 4.14 ELISA assay on cell death in hippocampus of SHR and WKY rats at 15 days after sham or ischemia operation. After sham or ischemia operation at 15 days, the rats were anesthetized by chloral hydrate and killed, then the brains were removed and the proteins of hippocampus were extracted and quantified. Cell death of hippocampal cells was determined at 405nm by ELISA method. Data were represented as mean ± SD, Sign + indicates significant difference with $p<0.05$ from the respective sham groups.

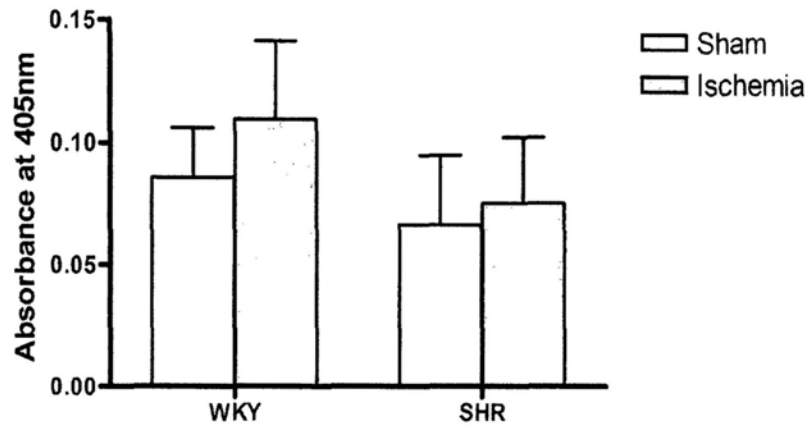


Figure 4.15 ELISA assay on cell death of cerebellum in SHR and WKY rats at 15 days after sham or ischemia operation. After sham or ischemia operation at 15 days, the rats were anesthetized by chloral hydrate and killed, the brain was removed and proteins of the cerebellum were extracted and quantified. Cell death of cerebellum was determined with ELISA method. OD was measured at 405 nm. Data were mean ± SD.

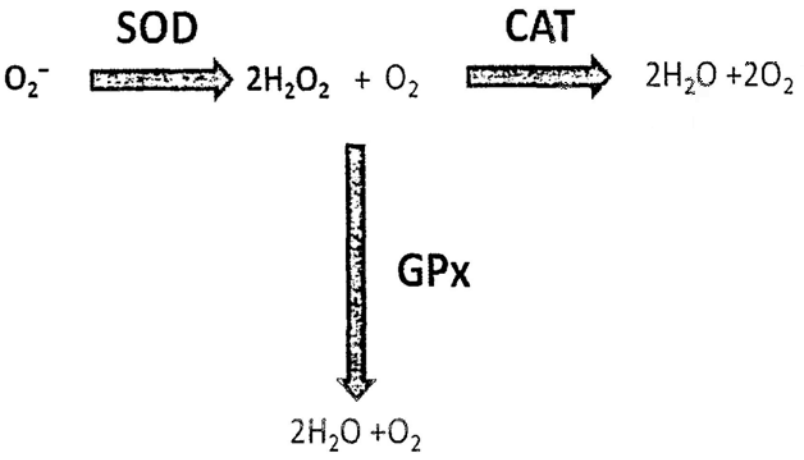


Figure 4.16 Cellular pathways involved in the elimination of the free radicals superoxide (O_2^-) and hydrogen peroxide (H_2O_2), showing how superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are involved in antioxidation.

Chapter 5 The preventive effects of Pien Tze Huang in chronic brain ischemia with co-existed hypertension

5.1 Introduction

Previous studies on cerebrovascular diseases had confirmed that hypertension and intracranial atherosclerosis are the main causes of cerebrovascular stenoses (Li, et al., 2005; Liu, et al., 2005), which can cause low perfusion of the brain. Eventually the chronic low perfusion might bring about irreversible damages. The preventions and improvements of low perfusion of the brain are the keys to prevent serious brain damages (Adams Jr, et al., 2003).

Spontaneously hypertensive rat (SHR) were bred from Wistar rats and this kind of rats has a high incidence rate of hypertension (Okamoto and Aoki, 1963). Commonly, the hypertension would occur when rats are 16 weeks old. SHR shows the same mechanism and pathological changes of target organs and complications as those in humans (Takahashi and Smithies, 2004). SHR is the most widely used animal model to study human primary hypertension and in the screening of possible therapeutic drugs. In this study, the two (left and right) carotid arteries of SHR were occluded to induce cerebral ischemia in concurrence with the existing hypertension, a condition which is more akin to the human situation (Yamori, et al., 1976b).

Functional magnetic resonance imaging (fMRI) is an imaging technique for detecting changes in metabolic activities based on the blood-oxygenation-level-dependence (BOLD) effect (Bock, et al., 1998). Blood flow of different brain areas correlates with cortical activations and thus metabolic activities. fMRI is precise spatially and

one can simultaneously obtain anatomical and functional images (Logothetis, et al., 1999). Therefore, it is widely applied to the studies of brain activities (Koshino, et al., 2005).

Pien Tze Huang (PTH) is a precious traditional Chinese medicine with the effects of anti-inflammation, detoxification, analgesia and anti-edema (Lin, et al., 1985; Zhao and Pan, 2006; Meng and Gu, 2008). It is mainly used in the treatments of liver diseases and cancers (Xu and Yan, 2003). There is no report on PTH uses for nervous system diseases or neuroprotection. However, a number of studies suggested that some constituents of PTH, such as Radix Notoginseng, snake's gall, calculus bovis and natural musk might have various beneficial effects including protection of blood vessels, in particular the endothelial cells, and nerves, improvement of cerebral circulations, limiting oxidation, and inhibition of neurotoxicity (Kenarova, et al., 1990; Nah, et al., 1995; Han, et al., 1999; Li, et al., 2006a). It has also been widely used in the treatments of cardiovascular diseases. These effects led us to study whether or not PTH has neuroprotective activities.

In this chapter, we planned to investigate the therapeutic effects of PTH on chronic cerebral ischemia in rats that also suffered hypertension. ELISA and fMRI methods were used to study the pathological and functional changes in the brain. Any neuroprotective effects of PTH against these changes would provide basic experimental evidences for clinical application of PTH in brain ischemia.

5.2 Materials and methods

5.2.1 Materials

PTH was supplied by Zhangzhou Pien Tze Huang Pharmaceutical Co. Ltd. (Zhangzhou, Fujian, China). Cell death detection ELISA kit and other chemicals were obtained from the companies as listed in Chapter 4.

5.2.2 Animals

Eighteen male Wistar Kyoto (WKY) rats, aged 3 months, were randomly divided into 3 groups: sham operation, chronic cerebral ischemia and PTH treatment groups. There were 6 rats in each group. In the same way, 18 male SHR rats aged 3 months were divided into three groups. The methods for developing sham operation and chronic cerebral ischemia models have been described in details in Chapters 3 and 4. In the PTH treatment group, before bilateral common carotid artery (CCA) ligation, rats had intragastric administration of PTH at a dosage of 18 mg/kg body weight/day for 3 months. The rats in chronic cerebral ischemia group had intragastric administration of 0.9% saline for 3 months before operation. The rats in the sham operation group were not infused intragastrically. Fifteen days after the operation, the rats in all groups, both WKY and SHR, were sacrificed by cervical dislocation, and brain tissues were immediately removed. The hippocampus and cerebellum were identified and excised. The hippocampus was chosen as it was most susceptible to ischemia induced by the bilateral CCA ligation; the cerebellum was a kind of control because its blood supply comes from the vertebral-basilar artery, which was not ligated. The specimens were snap frozen in liquid nitrogen and stored at -80 ^{o}C untill the cell death assay. In addition, another 15 male SHR of 3 months old were divided into 3 groups, sham operation, chronic cerebral ischemia and PTH treatment groups (each group had 5 rats), and were treated in the same way as described above. These

rats were used for brain fMRI experiment 15 days after the bilateral ligation of the CCA or sham operation.

5.2.3 Cell death detection

ELISA was used; the procedures were the same as those described in Chapter 4.

5.2.4 Functional magnetic resonance imaging

Rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), and put in a prone position and fixed. MRI scan was performed using appropriate coils. Then the tails of rats were stimulated by placing upon it different weights, while the scan was performed. The detailed procedure of fMRI is described as follow:

Two different weights were used as tail stimuli. A weight of 500 g was defined as the light stimulus and 1000 g as the heavy stimulus. We used "off-on" as the stimulation mode. Each stimulation cycle was performed according to the following sequence: a baseline scan of 4 seconds, a rest period of 10 seconds (off), followed by a 10 seconds of stimulation (on) with weight. For each stimulus, the "off-on" mode was repeated three times. The process was performed in the same manner for both light and heavy stimuli. For fMRI data acquisition, an echo planar imaging (EPI) pulse sequence was used (TR = 2000 ms, TE = 33 ms, slice thickness = 0.8 mm, slice gap = 0 mm, FOV = 60 mm, matrix = 64*64, NSA = 1). In each scan, 12 coronal slices (images) were recorded. In total 64 acquisitions were obtained for each fMRI experiment. The fMRI images were analyzed with ViewForum workstation (Phillips

Medical System, Best, Netherlands). The total volume of the activated region in the rat brain was calculated.

5.2.5 Statistical analysis

All data were presented as mean ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by a post hoc comparison test using Fisher's LSD test or independent t-test to evaluate the differences between groups for significance. The signed rank test was used to compare the difference of activated region between light and heavy stimulation of different groups in fMRI experiments. $p \leq 0.05$ was considered as statistically significant. All statistical analyses were performed using SPSS 16.0.

5.3 Results

5.3.1 Cell death in hippocampus and cerebellum

We evaluated the cell death in the hippocampus of 3 groups of WKY and SHR after ligation or sham operation. For the hippocampus, both chronic cerebral ischemia WKY and SHR groups had significantly higher cell death than their respective sham operation groups ($p < 0.05$) (Figure 5.1). Importantly, cell death in the PTH treatment WKY and SHR groups showed no difference as compared to their respective sham operation groups; PTH could significantly decrease the cell death in the hippocampus in cerebral ischemia ($p < 0.05$) (Figure 5.1). In other words, PTH protected hippocampus from damages resulted from either cerebral ischemia only or cerebral ischemia and hypertension together. For cerebellum, there were no

significant differences between the sham operation, chronic cerebral ischemia and PTH treatment groups (Figure 5.2).

5.3.2 Functional magnetic resonance imaging

We used SHR for fMRI experiment and found that both the chronic cerebral ischemia and PTH treatment groups displayed larger activated volumes in reaction to the tail stimuli as compared to the sham operation groups (Figure 5.3). The activated volume was the highest in the chronic cerebral ischemia group followed by the PTH treatment group then sham the operation group. However, the increases in activated volume in the light (500 g) stimulation was statistical significance ($p < 0.05$) only between the chronic cerebral ischemia or the PTH treatment groups and the sham operation group. Figure 5.4 showed representative fMRI scans of the rat brain when the tail of rat were stimulated with 500 g weight.

5.4 Discussion

In Chapter 4, our results showed that the numbers of apoptotic cell and the amount of cell death by TUNEL and ELISA methods in the hippocampus from the cerebral ischemia hypertension group were significantly higher than those in the hypertension only or cerebral ischemia only groups However, in the cerebellum, there was no significant difference among the groups. In this Chapter, we used ELISA method to study cell death in the hippocampus and the cerebellum 15 days after ischemia in SHR, i.e. chronic cerebral ischemia with existing hypertension, and in WKY, i.e. chronic cerebral ischemia without hypertension, and tried to evaluate any effects of PTH. In the PTH treatment group, the cell death in the hippocampus was reduced to

a similar levels as the sham operation group, whereas the chronic cerebral ischemia group showed significant increases in cell death ($p < 0.05$) (Figure 5.1). This suggested that PTH could significantly reduce brain cell death in cerebral ischemia with and without co-existing hypertension. On the other hand, there was again no significant difference between the cerebellums of the different groups (Figure 5.2); this confirmed the TUNEL results from Chapter 4. It might be suggested that PTH did not have any protective effect in cerebellum, but in fact the cerebellum was less ischemic and there was little cell death in the first place. This was because the cerebellum was supplied by the vertebral arteries, which were not occluded, whereas the hippocampus was supplied by the common carotid arteries, which were occluded.

fMRI is a noninvasive tool based on blood-oxygen-level-dependent (BOLD) contrast (Bock, et al., 1998). It is used for mapping brain metabolic activity by measuring the change in cerebral blood flow and oxygenation. BOLD therefore reflects the degree of tissue oxygenation. The stimulus to neuron cells resulted in an increase of blood flow, which delivered more oxyhemoglobin and less deoxyhemoglobin. Hence, stronger signals would be detected at areas with increased metabolic activity (Detre and Wang, 2002).

Our results showed that a lighter 500 g stimulus to the tails of SHR significantly increased the activated brain volume in the chronic cerebral ischemia group and PTH treatment group than in the sham operation group. This probably reflected a compensatory mechanism of using more areas to do the same task in the chronic ischemic brain (Figure 5.3 and 5.4). However, no statistical significance was found among the 3 groups under a heavier stimulus of 1000 g. One possible reason for this may be a heavier stimulus would trigger not only proprioceptive and tactile

responses, but also responses of other modalities and therefore the end results could be complicated.

The two PTH treatment groups of SHR in the cell death assay and fMRI experiment showed discrepant results; the differences from the sham operation group were significant in cell death results for hippocampus, but not in fMRI results. The reasons could be two-fold. First, the cell death results were region specific - the differences were significant for the hippocampus but not for cerebellum. fMRI tested the whole brain, so significant difference in one region may not be reflected in the overall result for the whole brain. Second, the cell death assay tested the extent of dead cells, and fMRI tested the loss of functional integrity which could be resulted from cell death or injry. PTH treatment protected and decreased the number of dead cells, but might not be able to prevent the brain cells from injury or losing certain functions, and the loss of functions probably resulted in reduced signal intensity. Nonetheless, it is noted that the activated volume was the highest in the chronic cerebral ischemia group followed by the PTH treatment group and lastly the sham operation group, although the differences were not significant. This suggested that PTH treatment could decrease the damage in functional integrity but not to the extent of statistical significance.

Taken together, our results strongly suggested that PTH could protect brain damage from ischemia with neuroprotection effects. Previous studies have showed that Panax notoginseng saponins (PNS), the major active component of Radix Notoginseng in PTH, could effectively decrease the level of endothelin in the plasma of patients with cerebral infarction (Li, et al., 2000). It could also inhibit the proliferation of vascular smooth muscle cells and lower the level of blood lipids (cholesterol and triglycerides)

(Chen, et al., 1984; Kenarova, et al., 1990; Li, et al., 1999). Muscone, an active substance of musk in PTH, could reduce the level of endothelin in the plasma of stroke-prone renovascular hypertensive rats and improved the vascular remodeling caused by hypertension (Li, et al., 2006a). Therefore, both PNS and muscone could protect blood vessel endothelium resulting in less vasogenic brain damage.

The dysfunction of energy metabolism is a major factor that induces brain damage during cerebral ischemia (Zhang, et al., 2001). Calculus bovis raised the level of glucose in the extracellular fluid of the hippocampus, and lowered the concentration of lactate. Both actions are protective to brain cells (Cao, et al., 2008). In cerebral ischemia, brain cells could produce a large amount of free radicals and unsaturated fatty acids and membranes could be oxidized by these free radicals. Free radicals and malondialdehyde (MDA) produced during lipid peroxidation would damage the cell membrane and biological macromolecules inside the cells and may lead to cell death. Therefore, the MDA level and superoxide dismutase activity (SOD) could indirectly indicate the degree of cell damage and the ability to eliminate free radicals (Clarkson, 1995; Karlsson, 1997; Rudnicki, et al., 2007). PNS and ginsenoside Rgl, PNS's monomer, as well as bile pigment and taurine, which are the major components of muscone and calculus bovis, raised the SOD activity and decreased the MDA levels in the ischemic brain tissue and plasma. Hence these substances could reduce brain damage during cerebral ischemia (Zhu, 1997; Chang, et al., 1999). Furthermore, both PNS and taurine inhibited Ca^{2+} channels and prevented the overload of Ca^{2+} in nerve cells in the brain damage (Nah, et al., 1995; Han, et al., 1999; Guan, et al., 2006; Wu, et al., 2009). Muscone also could decrease the content of excitatory amino acids (EAAs) and the expression of N-methyl-D-aspartic acid (NMDA) receptor in the

brain tissue; this could also alleviate the overloading of Ca^{2+} and reduced the damage (Liang, et al., 1996; Sun, et al., 2009). All these actions could minimize the damage to the brain's blood vessels and reduce the intraluminal pressure (Nah, et al., 1995). All together, through different mechanisms, PTH could protect brain cells and reduce the cell death. The protective effects of PTH, as we had observed here, could be a combined result of these mechanisms.

5.5 Conclusion

PTH has some protective effects on the brain in chronic brain ischemia induced under either normotensive or hypertensive conditions in rats. To a certain degree, PTH might enhance the protection on the functional integrity of the brain under such adverse conditions.

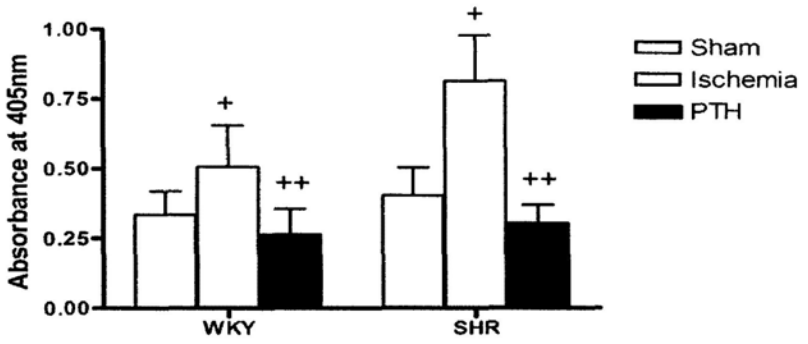


Figure 5.1 ELISA assay on cell death of hippocampus in sham operation, chronic cerebral ischemia and PTH treatment groups of WKY and SHR, 15 days after the ligation operation. In both WKY and SHR, ischemia significantly increased the cell death as compared to the sham operation groups (+ indicates significant difference, $p < 0.05$) PTH protected hippocampus from the damage induced by ischemia and showed significant decreases (++ indicates significant difference, $p < 0.05$) as compared to the ischemia groups.

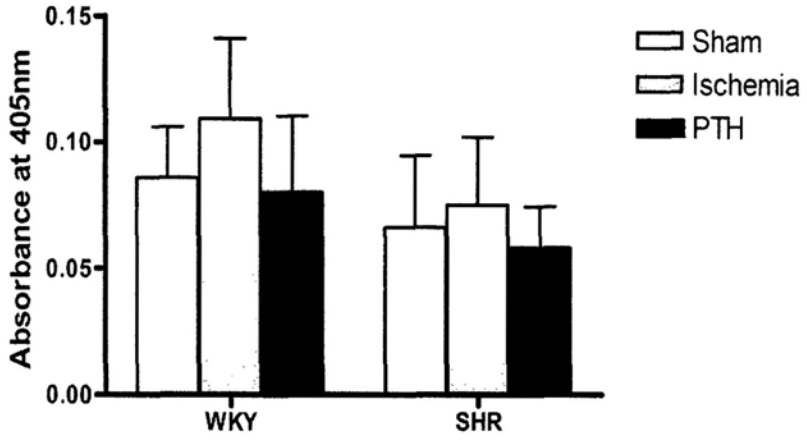


Figure 5.2 ELISA assay on cell death of cerebellum in sham operation, chronic cerebral ischemia and PTH treatment groups of WKY and SHR 15 days after the ligation. There were no significant differences among the 3 groups of WKY and SHR.

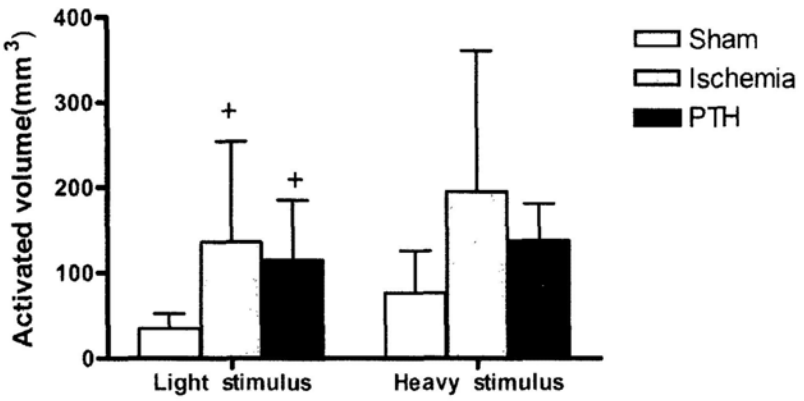
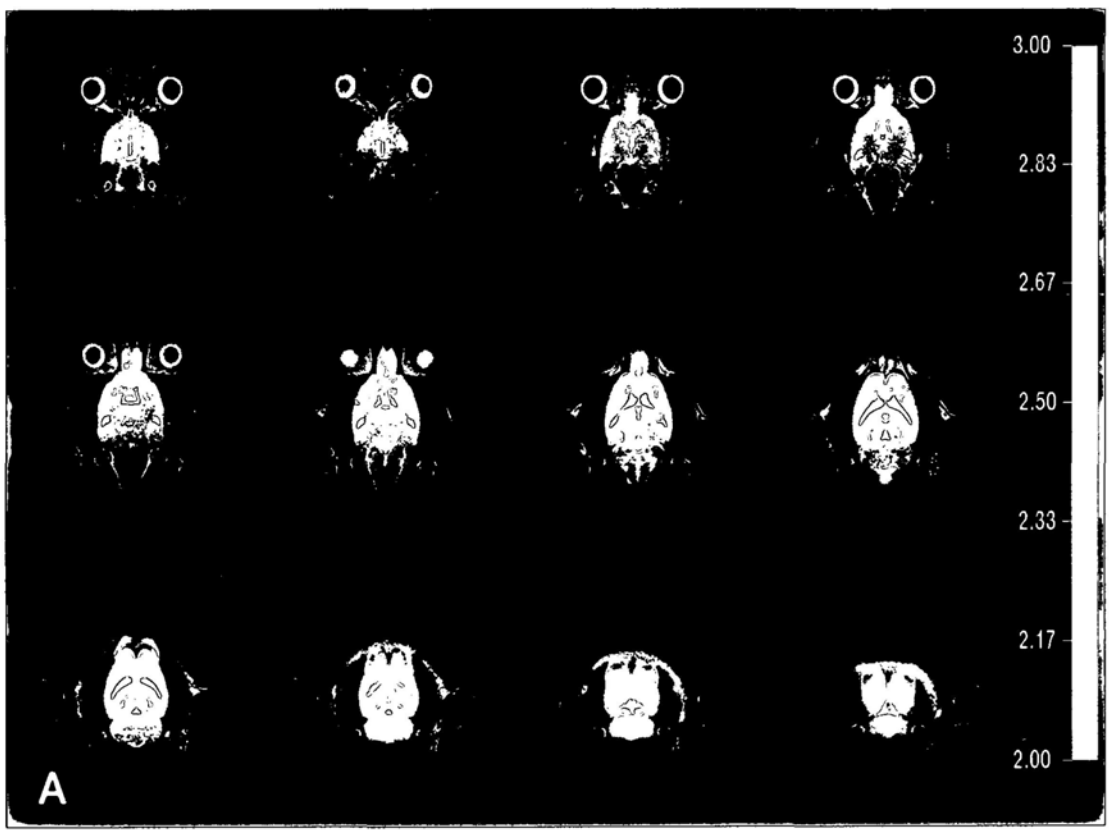


Figure 5.3 fMRI analysis of brain activations in sham operation, chronic cerebral ischemia and PTH treatment groups of SHR 15 days after the ligation. The rats were anesthetized by ketamine and xylazine, and placed into the MRI instrument. fMRI scans were acquired during two different tail stimuli (placement of a 500 or 1000 g on the tail respectively). Data were means ± SD of activated volumes of of brain regions. Sign + indicates significant difference with $p < 0.05$ from sham operation group.

Figure 5.4 Images of fMRI serial scans of 6-month old SHR with tail stimulus of 500g weight. Each image represented one scanned section of the rat brain; the red region (scale: 2.83-3.00) was the activated brain area when the tail was stimulated. **A**, **B** and **C** represent the scans from three rats, one from each of the sham operation, chronic cerebral ischemia and PTH treatment groups respectively. The activated areas (the red regions) were larger in the chronic cerebral ischemia rat (**B**) than in the other two rats showed in (**A**) and (**C**).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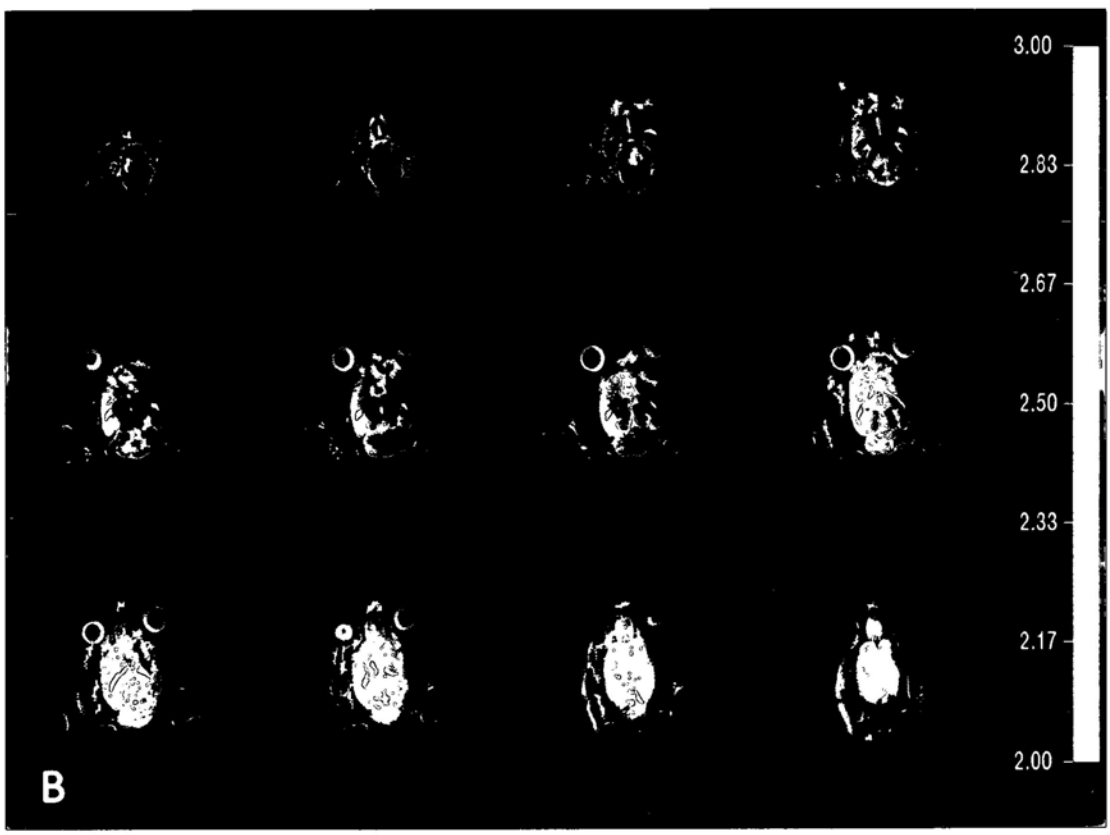
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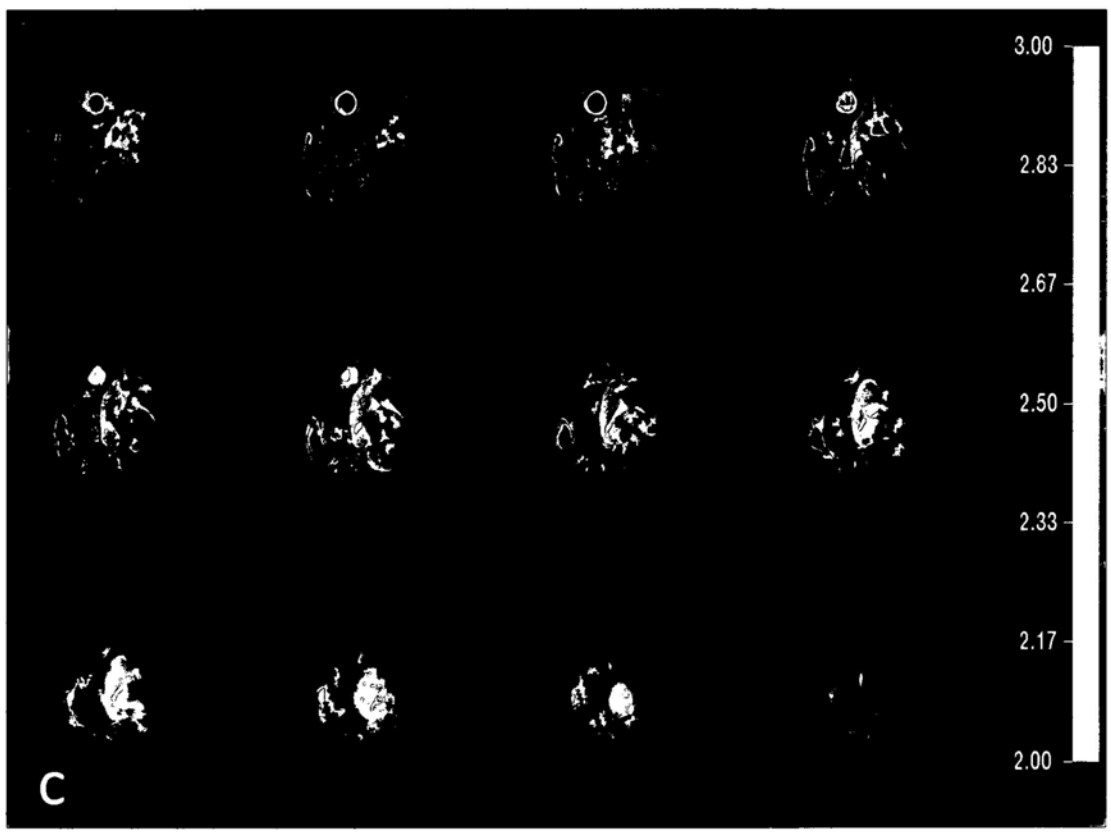
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Chapter 6 The preventive effects of Pien Tze Huang in stroke

6.1 Introduction

Stroke carries high fatality and high rate of disability (Thorvaldsen, et al., 1995; Feigin, 2005) and yet there is no established effective treatment. Before the onset of the disease, most patients usually had hypertension and/or brain atherosclerosis for quite some time, which subject the brain to a chronic low perfusion state (Li, et al., 2005; Liu, et al., 2005). Therefore, preventing the development such conditions to stroke are the key means to reduce subsequent disability and fatality of the patients. This chapter explores the possibility of employing Chinese medicine for the treatment or prevention of the disease.

Stroke-prone spontaneously hypertensive rats (SHRsp) were bred from spontaneously hypertensive rats (SHR), which usually died of stroke by Yamori in 1970. Six weeks after birth, the blood pressures of SHRsp begin to rise, and reach 200 mmHg systolic by 10-15 weeks, and peak at 20-24 weeks at above 200 mmHg. The life expectancy of male SHRsp is 9 months for male, and 12 months for female. Typical of this strain, 100% of the rats have hypertension and 80% of them develop stroke. At present, SHRsp is recognized as the stroke animal model, because its symptoms and brain pathology are similar to those in humans (Okamoto, et al., 1986).

Pien Tze Huang (PTH) is a rare traditional Chinese medicine with the effects of anti-inflammation, anti-edema, detoxification and analgesia (Lin, et al., 1985; Zhao and Pan, 2006; Meng and Gu, 2008). Currently, it is mainly used in the treatments of hepatopathy, cardiovascular diseases and cancers. Up to this day, there are few

reports about the PTH effects on the nervous system and neural protection. A number of studies have, however, suggested that the main constituents of PTH, such as Radix Notoginseng, snake's gall, calculus bovis and natural musk, had various protective functions on blood vessels and nerves. For instance, they could protect endothelium, improve blood circulation, enhance cellular resistance to oxidation and resistance to neurotoxic agents (Kenarova, et al., 1990; Nah, et al., 1995; Han, et al., 1999; Li, et al., 2006a). In fact, PTH has already been widely used in the treatments of cardiovascular diseases. Because stroke is a vascular problem that results nerve cell damage, it would be useful to find out whether PTH would also exert these protective effects in the brain.

In this study, SHRsp were fed with PTH before the usual time of the onset of stroke for investigation of the protective effects of PTH on hypertension-related brain stroke damages.

6.2 Materials and methods

6.2.1 Materials

Rat B-cell leukemia/lymphoma-2 (Bcl-2) and Rat Bcl-2 associated X protein (Bax) ELISA Kits were purchased from CUSABIO BIOTECH Co. Ltd. (Catalog No. CSB-E08854r and CSB-E12151r, Newark, NJ, USA). Pien Tze Huang, cell death detection ELISA kit, DC protein assay kit and other chemicals used were the same as those described in Chapter 4.

6.2.2 Animals

Totally 44 6-week SHRsp were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Haidian District, Beijing 100089, P.R. China) in this study. Rats were kept under conventional conditions in the Laboratory Animal Services Centre of the Sun Yet-Sen University, Guangzhou, China. Standard diet and water were given *ad libitum*. All experiments were conducted according to the guidelines of the National Institute of Health Guide for the care and use of laboratory animals and their uses were approved by the Animal Ethics Committee of the Sun Yet-Sen University.

All the rats were given 1 week to adapt to the experimental environment. Then the animals were randomly divided into two groups: normal saline treated SHRsp group (n = 30) and PTH treated SHRsp group (n = 14), which were infused intragastrically with normal saline and PTH suspension in normal saline (18 mg/kg body weight/day) respectively. Body weights of all the rats were recorded at the beginning when PTH or saline were given. The neurologic status of each rat was evaluated every day carefully by an observer who had no knowledge of the grouping. A grading scale of 0-4 was used to assess the rat's activities (Kaneko, et al., 1985) and was related to the severity of stroke (Table 6.1). Scores 1 and 2 were regarded as having mild stroke, and scores of 3 and 4 as severe stroke. The rule was if a rat exhibited the appropriate behavior at one step but not at the subsequent step, it was graded as the former. The score was made at 9:00 and 15:00 every day. When stroke was first noticed, the score was recorded. Once the stroke was confirmed, feeding of saline or PTH would be terminated. Then the symptomatic and physical changes were recorded for the remaining days until the death of the rats. The rest of the rats that did not have stroke were kept until the end of the experiments at the end of the seventeenth week. Rats

were all killed by cervical dislocation, the brain was removed from the skull and the hippocampus and cerebellum were dissected out and stored at -70 °C until the analyses.

6.2.3 Cell death ELISA

Experimental methods were the same as those described in Chapter 4.

6.2.4 ELISA for Bcl-2 and Bax

For the expression levels of Bcl-2 and Bax, 100 mg (wet weight) of brain tissue sample was homogenized with a glass homogenizer in 0.5 ml of 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 0.1 mg/ml PMSF, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, and 5 μg/ml aprotinin. The homogenate was centrifuged at 14,000 rpm for 30 minutes at 4 °C and the supernatant obtained was either immediately used or stored at -80 °C until analysis. The protein concentration of the extract was determined by the DC protein assay. Equal amount of standard (100 μl) or sample protein (50 μg) was added into each of the wells of the ELISA plate, and incubated for 2 hours at 37°C. Then the supernatant of each well was removed and 100 μl of Biotin-antibody working solution was added and further incubated for 1 hour at 37 °C. The solution was removed by aspiration and the cells were washed three times with Wash Buffer (200 μl) and then the Wash Buffer was discarded by inverting the plate and blotting it against clean paper towels. Then 100 μl of HRP-avidin was added to each well; the plate was covered and incubated for 1 hour at 37 °C. After aspiration and three other washes, 90 μl of 3',5,5'-tetramethylbenzidine (TMB) substrate was added to each

well and incubated for 30 minutes at 37 °C in the dark. Then 50 μl of Stop Solution was added to each well and the solutions were mixed by gently tapping the plate. Finally, the optical density (OD, or absorbance) of the sample in each well was measured at 450 nm using a microplate reader. A standard OD curve was constructed and the values from the samples were normalized. The data of the samples were linearized by plotting the log of the Bcl-2 or Bax concentrations versus the log of the OD readings and the best fitting line was determined by regression analysis. If the samples had been diluted, the concentration read from the standard curve would be multiplied by the dilution factor. The relative amount of the target protein was calculated as OD relative to that of the standards.

6.2.5 Statistical analysis

All data were presented as mean ± standard deviation (SD). Statistical analysis was performed by Chi-square test or independent t-test to evaluate the differences between the groups using SPSS 16.0. When p-value was less than 0.05, the difference was considered statistically significant.

6.3 Results

6.3.1 Comparison of the incidence, severity and time of death of stroke in PTH and saline fed SHRsp

From the beginning of PTH or saline feeding, the two groups of SHRsp rats were closely observed. The day from the beginning to the day of the onset of stroke of each rat was recorded and given a score according to the assessment criteria of stroke

for rats (Kaneko, et al., 1985). All the stroke-stricken rats were weighed three times every day until death. The data showed that the earliest days when stroke occurred was 38, the latest was 73 days, and the intermediate time was 50 days. We divided all rats into two groups according to the time when stroke occurred those having stroke at or earlier than 50 days, and those having stroke later than 50 days (Table 6.2). We found significantly more PTH fed rats had stroke after 50 days, while more control (saline fed) rats developed stroke before 50 days ($p = 0.05$). So it may be suggested that PTH might have a protective role against stroke occurrence.

We compared the severity of stroke between PTH or saline fed rats using the scheme for rating the deficits of the stroke-stricken rats (Table 6.1) (Kaneko, et al., 1985). The results showed that PTH did not affect the severity of stroke since there was no significant differences between the number of PTH and saline fed rats that developed mild or severe stroke deficits (Table 6.3). Although PTH showed no significant preventive action to reduce the severity of stroke, PTH could significantly delay the time of death of stroke ($p = 0.047$), since significantly fewer PTH treated rats died before or at 50 days than after (Table 6.4); compared with the saline-treated rats, more PTH treated rats survived beyond 50 days.

6.3.2 Cell death in hippocampus and cerebellum

In this part, we evaluated cell death by ELISA method in the hippocampus and cerebellum samples obtained from the SHRsp when they died. As shown in Figures 6.1A and B, the optical densities from both the hippocampus and cerebellum of the PTH group were significantly lower than those of the saline group ($p < 0.05$); this showed that there was less cell death in the PTH group. Hence PTH feeding might

have a significant protective role against cell death. Comparing between the PTH and saline groups, the hippocampus showed no significant difference in the Bcl-2/Bax ratio (Figure 6.1C); however the cerebellum showed a significantly higher Bcl-2/Bax ratio ($p < 0.05$) in the PTH group than the saline group (Figure 6.1D).

6.4 Discussion

SHRsp has been well recognized as the animal model for stroke study. The stroke symptoms and brain pathology occur as early as in the sixth week postnatal and are similar to those in humans (Yamori, et al., 1976b). The strokes are commonly ischemic stroke, and hemorrhagic stroke is relatively scarce.

A number of studies (Ru, et al., 2008) have suggested that effective control of blood pressure was the key to reduce the chance of having stroke and its severity. Our results showed that the onset of stroke was significantly delayed in the PTH group; and the subsequent death in this group was also delayed (Tables 6.2 and 6.4). Also there was a decreasing trend in the severity of stroke in the PTH group compared with that in the saline group (Table 6.3). These may be related with the hypotensive effects of the active ingredients of PTH. Indeed, panaxatriol saponin (PTS), one of the vascular active ingredients of PTH, could reduce blood pressure and stroke incidence in stroke prone renovascular hypertensive rats (RHRSP) (Zhao, et al., 2006). Taurine,one of the active substances of bezoar and snake bile, could also reduce blood pressure and delay the development of stroke in SHR (Gao, et al., 2008). The anti-hypertension mechanism of PTS and taurine were related to calcium dependent channel blockage and dilatation of blood vessels (Zhao and Ruan, 2007). Muscone also has showed a certain anti-hypertension function (Li, et al., 2006a).

Although PTH seemed unable to prevent the onset of stroke in SHRsp and the progression, PTH feeding might delay the time of onset of stroke and the time of death. This may be significant as SHRsp are highly prone to develop stroke, and in fact in our study all saline fed rats developed stroke. Therefore, this preventive effect of PTH would be more pronounced in normal rats, and probably in humans as well.

There are two types of cell death in the pathology of stroke, necrosis and apoptosis. Generally, necrosis occurs in the case of severe damage, and less severe or mild damage leads to apoptosis (Srinivasan, et al., 1996). When stroke occurs, necrosis will appear at the center of the cerebral ischemic area, while apoptosis will appear around the necrotic tissue at the periphery (Danielisova, et al., 2004; Shin, et al., 2004). The cell death was measured using cell death ELISA (total cell death) in this study. Our results showed that PTH could significantly decrease the cell death in both the hippocampus and cerebellum as compared to control saline (Figure 6.1A and 6.1B). This strongly suggested that PTH would have some protective effects against cell death in both brain regions. The question is, which type of cell death was protected by PTH?

Bax is one of the well-known pro-apoptotic molecules, and it regulates apoptosis by interacting with Bcl-2, which inhibits apoptosis and is located in the mitochondrial membranes (Krajewski, et al., 1994). Internal signals of cell damage cause oligomers of Bax to insert into the outer mitochondrial membrane triggering cytochrome c release to initiate apoptosis. On the other hand, Bcl-2 can complex with Bax in such a way that the release of cytochrome c is inhibited and thus apoptosis is prevented (Oltvai, et al., 1993). The Bcl-2/Bax ratio - an anti-apoptotic to apoptotic index, is therefore a crucial factor in determining the progress of apoptosis (Woo, et al., 2000;

Raghupathi, 2004). This ratio of Bcl-2/Bax may also be the key factor to determine the type of cell death - whether it will be necrosis or apoptosis (Woo, et al., 2000). The higher is the Bcl-2/Bax ratio, the more preponderant will be the anti-apoptotic activities (Oltvai, et al., 1993). In our study, the ratio of Bcl-2/Bax in the cerebellum of the PTH fed rats were significantly higher than that of the saline fed rats, but in the hippocampus there was no difference between the two groups (Figure 6.1). This suggested that although cell death occurred in both the hippocampus and the cerebellum of SHRsp after stroke, the major form of cell death may be different.

In brain injuries, the preponderance of necrosis over apoptosis indicates severe ischemia. Hippocampus is one of the most sensitive areas to ischemia (Sakurai-Yamashita, et al., 2003). In our cerebral ischemia animal models, the type of cell death in the hippocampus was likely to be mainly necrosis, because the common carotid arteries, which were ligated, were the blood supply of the hippocampus. On the other hand, the type of cell death in the cerebellum could be both necrosis or apoptosis, or mainly apoptosis, because it was supplied by the vertebral arteries and not the occluded carotid arteries. It might be that in strokes of the SHRsp rats, the common carotid arteries were more prone to occlusion than the vertebral-basilar arteries, as seen in the following discussion.

Our results showed that PTH increased the Bcl-2/Bax ratio in the cerebellum, which indicated that apoptosis would be inhibited (Figure 6.1D), while significantly more cell death did occur as compared with the saline group (Figure 6.1B) suggesting that the cell death was due to both necrosis and apoptosis. On the other hand, PTH did not change the Bcl-2/Bax ratio in the hippocampus of the PTH group (Figure 6.1C),

while significantly more cell death would be due to a majority of necrosis (Figure 6.1A).

In the future study, one should perform comparative morphological studies on the hippocampus and cerebellum to confirm the type of cell death. Importantly, our results showed that PTH could prevent apoptosis by increasing the Bcl-2/Bax ratio but not necrosis in brain in rat.

The next question is, how does PTH protect brain cells from apoptosis by increasing the Bcl-2/Bax ratio. Obviously this warrants further studies. Nonetheless, we could examine the functions of the PTH's ingredients that might be related to neuroprotection as reported in the literature. After stroke, the sudden interruption of blood supply would lead to energy metabolism disorders, which would suppress the activity of Na^+-K^+ enzymes in membranes, causing membrane depolarization followed by releasing of excitable neurotransmitters into the extracellular space. Voltage dependent and the N-methyl-D-aspartic acid (NMDA) receptor related Ca^{2+} channels would be excessively activated and this would lead to the opening of Ca^{2+} channels with an increase in Ca^{2+} inflow and an overloading of intracellular Ca^{2+}. This would disrupt cell functions and damage cell structures (Zuccarello and Anderson, 1989). Both Panax notoginseng saponins (PNS) and taurine in PTH have the functions of calcium channel blockage, which might prevent calcium overloading inside nerve cells in stroke (Zhao and Ruan, 2007). Furthermore, PNS can increase the expression of Bcl-2 in brain tissue after stroke, thus inhibiting apoptosis (Gu, et al., 2006). It can also inhibit the activation of caspase-3 in the brain tissue, thus stopping the cascade of apoptotic changes (Li, et al., 2006b).

Musk, another important ingredient has been found to significantly reduce the level of excitatory amino acids (EEAs) and the expressions of NMDA receptor in brain tissues, thus inhibiting the opening of Ca^{2+} channels and Ca^{2+} inflow and hence preventing Ca^{2+} overloading, and lessening the functional and structural damages (Liang, et al., 1996; Sun, et al., 2009). In addition the edematous changes were also reduced. In another study it was found that musk significantly increased the number of astroglial cells with positive expression of nestin and fibrillary acidic protein (GFAP) around the infarction area (Jiang, et al., 2007). The reactive gliosis in the ischemic area might protect neurons from the injuries, enhance the function recovery, and promote the proliferations of neural stem cells and revascularization. Beside these benefits, the active ingredients of PTH can protect blood vessels and nerves, and lessen brain damage by various means, such as protecting the endothelial cells improving the energy metabolism, scavenging free radicals to prevent oxidative damages, and improving brain circulations (Stocker, et al., 1987). Together, these studies provided evidences on the protective effects of PTH on brain cells against damages resulted from various kinds of insults, which might include ischemic stroke and hypertension. Our results confirmed this neuroprotective effect of PTH against apoptosis but not on necrosis. This explained the rather little therapeutic effect of PTH in the SHRsp experiment, in which PTH could not prevent the severity of stroke in these rats. PTH only has the effects of delaying the onset of stroke and time of death. As discussed above, this may be significant as SHRsp are highly prone to developing stroke, but in case of normal animals, this preventive effect of PTH would be more pronounced.

6.5 Conclusion

PTH might have preventive effects against stroke in SHRsp; PTH feeding might delay the time of onset of stroke and the time of death. This could be significant not only in SHRsp, which are highly prone to develop stroke, but also in normal rats or even humans. Our results clearly indicated that PTH could prevent significantly cell death in hippocampus and cerebellum and also apoptosis by increases in the Bcl-2/Bax ratio in cerebellum.

Table 6.1 Neurologic examination grading system of stroke

Normal	Grade 0	No observable deficits.
Moderate	Grade 1	Slight reduction of activities or mild excitation.
	Grade 2	Significant reduction of activities or irritability sthenia.
Severe	Grade 3	Unable to walk, depressed psychocoma.
	Grade 4	Unable to stand, limb paralysis or paralysis one side of the body.

Table 6.2 Comparison of the numbers of SHRsp that developed stroke in PTH and saline fed groups.

Time of stroke	< 50 days	≥50 days
PTH	6	8
Saline	22	8

$F = 3.831, p = 0.05$

Table 6.3 Comparison of the severity of stroke in PTH and saline fed SHRsp.

Grading of stroke	1~2 (mild)	3~4 (severe)
PTH	8	6
Saline	9	21

$F = 1.305, p = 0.253$

Table 6.4 Comparison of the time of death in PTH and saline fed SHRsp.

Time of death	< 50 days	≥50 days
PTH	3	11
Saline	16	14

$F = 3.960, p = 0.047$

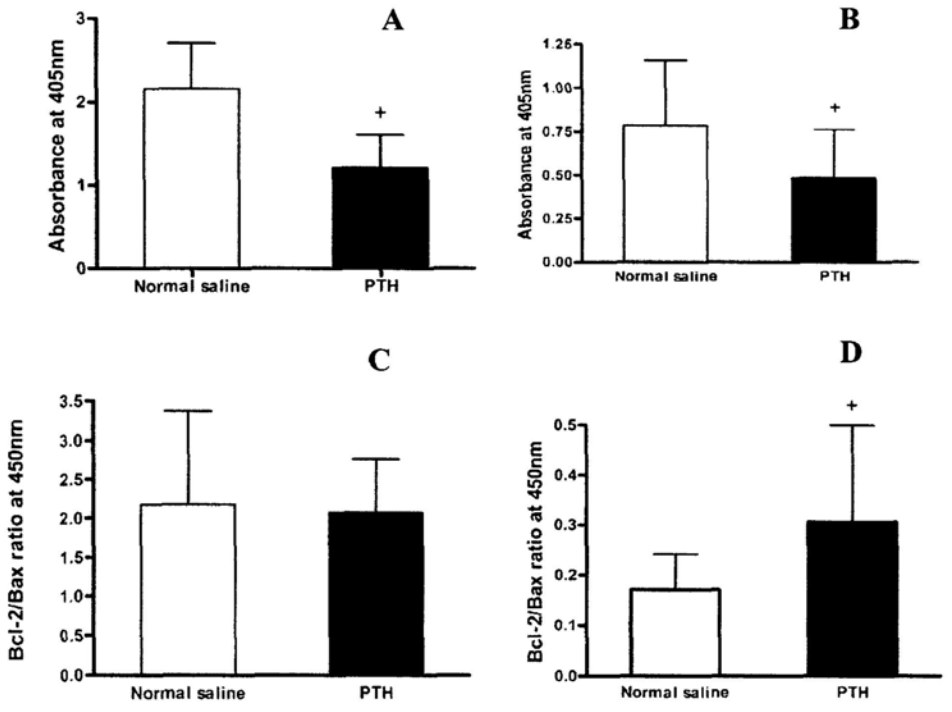

Figure 6.1 Cell death and Bcl-2/Bax ratios in hippocampus and cerebellum. **A**: Cell death in hippocampus in the PTH and saline groups. **B**: Cell death in cerebellum in the PTH and saline groups. **C**: The Bcl-2/Bax ratio in hippocampus in the PTH and saline groups. **D**: The Bcl-2/Bax ratio in cerebellum in the PTH and saline groups. Sign + indicates significant difference with $p < 0.05$ from the saline group.

Chapter 7 General discussion

7.1 Significance of the project

Stroke is one of the commonest diseases that threaten people's health. It can be classified into ischemic stroke and hemorrhagic strokes. The incidence of ischemic stroke is around 60%-80% of all strokes (Akopov and Cohen, 2003; Ovbiagele, et al., 2003). Ischemic stroke is more dangerous and extensive than hemorrhagic stroke. Therefore, our research has focused on ischemic stroke. As there are still no effective therapeutic treatments for stroke at present, the need to prevent it would be ever more pressing. Naturally, the prevention is to control the risk factors of stroke. Hypertension and cerebral arteriosclerosis are the most important risk factors of stroke, which often come together and increase the chance of cerebral ischemia (Li, et al., 2005; Liu, et al., 2005). At present, in clinical and basic research, hypertension and cerebral arteriosclerosis are usually regarded as independent factors in stroke. So, the establishment of a chronic cerebral ischemia animal model that has both cerebral arteriosclerosis and hypertension are more in keeping with the clinical reality.

Spontaneously hypertensive rat (SHR) is an ideal animal model to study human essential hypertension (Pinto, et al., 1998; DeLano, et al., 2006; Panico, et al., 2009), and rats with bilateral carotid occlusion has also been widely used to study chronic cerebral ischemia (Ni, et al., 1994).

Pien Tze Huang (PTH) is an expensive composite Chinese medicinal formula, and is used mainly for the treatments of liver diseases and cancer (Xu and Yan, 2003). However, a large number of recent studies have confirmed that the major

components of PTH, such as Radix notoginseng, snake bile, calculus bovis and musk have the apparent protective effects on nerves and blood vessels (Kenarova, et al., 1990; Nah, et al., 1995; Han, et al., 1999; Li, et al., 2006a). A major part of this project was to study the preventive role of PTH against brain damage induced by chronic brain ischemia combined with hypertension, as well as in stroke. This study will provide basic experimental data for further development of PTH and using PTH in other areas such as neurological disorders.

7.2 Overall discussion

In Chapter 2, patients with ischemic stroke from Northern and Southern Chinese mainland cities have been recruited for clinical analysis, which showed that 68.28% of patients have hypertension prior to the onset of the stroke. Compared with patients without hypertension, stroke patients with hypertension had significantly worse prognosis (p = 0.05). Our results were similar to previous study fifteen years ago (Cheng, et al., 1995) suggesting that there might not be gross changes in society environment or eating habit in the last 15 years. Previous studies have shown that hypertension was the most important risk factor of stroke; it could increase the incidence and severity of ischemic stroke in patients (Li, et al., 2005; Liu, et al., 2005). Our current investigations supported this conclusion. However, it is not clear how hypertension increases the development and severity of stroke.

The bilateral ligation of common carotid artery could cause a chronic ischemic state in the brain of SHR. The extent of the damage would depend on how much collateral circulation could be established in the corresponding brain areas. In order to determine the efficacy of our method, functional magnetic resonance imaging (fMRI)

(Bock, et al., 1998) was used to detect the changes in cerebral blood flow in the SHR and control Wistar-Kyoto (WKY) rats that had undergone surgical occlusion of the carotid arteries. The results in Chapter 3 showed that two kinds of rats showed a similar trend: fMRI blood-oxygenation-level-dependent (BOLD) signals disappeared in the 30 minutes after ligation, suggesting that blood flow had stopped or fell below detectable level but 1 hour after ischemia, the fMRI BOLD signals began to recover, though weaker than normal blood supply. This showed that cerebral blood flow was restored, but to a lower perfusion state. Cerebral blood flow was restored because the brain areas that were transiently deprived of blood supply, switched to the vertebral-basilar artery through the circle of Willis for supply (Sarti, et al., 2002). The blood supply of the vertebral artery system showed a corresponding reduction due to this compensatory change.

In Chapter 4, Nissl staining, TUNEL staining, ELISA and biochemical methods were used to detect the morphology, cell apoptosis, anti-oxidation activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and cell death in the hippocampus and cerebellum of SHR and control WKY rats at different times after the carotid occlusion. The results showed that hippocampal CA1 pyramidal cells were disoriented, and a large number of apoptotic cells appeared at 15 days after the occlusion. However, the structure of this region returned to normal at 30 days and 60 days after ischemia. In general, SOD, CAT and GPx showed high activities in the 15-30 days but had decreased by 60 day. In the normotensive WKY rats, the damage in the hippocampal CA1 area progressed more slowly.

In cerebral ischemia, brain cells produce a large amount of free radicals, and unsaturated fatty acids in the membranes could be oxidized by these free radicals.

Free radicals and malondialdehyde (MDA) produced during lipid peroxidation can damage the structure of cell membrane and biological macromolecules inside the cells leading to possible cell death (Huie and Padmaja, 1993). Our results shows the damage was particularly severe when chronic cerebral ischemia worked in combination with hypertension.

In Chapters 5 and 6, the preventive therapeutic activity of PTH against chronic cerebral ischemia was studied in rats with hypertension and stroke. Our results indicated that there was obvious decrease in the number of dead cells in PTH fed group in brain ischemia combined with hypertension. The fMRI data showed the volume of brain tissue activted with tail stimulation decreased in PTH fed group. Although the difference was not statistically significant, the data still may suggest PTH could lower the degree of damage induced by chronic brain ischemia combined with hypertension. It also points to the possibility that PTH may delay the onset of stroke and lower the severity of injury in SHRsp rats. This was proved in the last experiment of this project, which showed a reduction in the number of dead cells in the hippocampus of stroke-stricken rats.

7.3 Problems

(1) The rat ischemia model induced by the bilateral ligation of common carotid artery is commonly used in the study of chronic ischemia, but the acute ischemia after ligation of the arteries can damage brain tissue. This immediate effect may affect the subsequent development of the chronic conditions.

(2) From previous studies, the Wister rat, Sprague-Dawley rat and Wistar-Kyoto rat were the most widely used animals to investigate the effects of chronic brain

ischemia produced by bilateral carotid ligation, however the reported pathological changes and the extents of injuries were not always consistent (Ulrich, et al., 1998; Otori, et al., 2003; Choy, et al., 2006). The discrepancies suggested that different species and strains had different tolerance to ischemia. Even if the same species and strains were used, there might be different reactions because of different experimental conditions. For example, the use of different anesthetic agents can cause different autonomic nervous responses and brain metabolic rates (Miura, et al., 1998; Nellgard, et al., 2000). To produce a combined hypertension-ischemia model, more research will be needed to establish its anatomical, physiological and neurological characteristics.

(3) In clinical and basic research, our investigations have come to the same conclusion: the brain damage induced by chronic brain ischemia combined with hypertension was more severe than that induced by chronic cerebral ischemia alone, (see Chapters 2 and 4). In Chapter 3, the results showed that after the bilateral ligation of the common carotid artery, changes of cerebral blood flow in SHR and WKY rats showed a similar trend. This suggested that the two strains of rats might have similar degree of brain ischemia. That is to say, the cerebral ischemia combined with hypertension is a more complex situation than the simple ischemia.

7.4 Future studies

To further study the problems discussed above, the following studies may be proposed:

(1) To confirm the acute pathological changes in brain tissue immediately after permanent bilateral occlusion of the common carotid artery in SHR rats, and the relationships between such early changes with later changes in the chronic ischemic phrase.

(2) To expand the breadth and depth of study on the animal models of chronic cerebral ischemia with hypertension, on the pathological changes and the mechanisms of cell response to injuries in various regions of the brain.

(3) To further study the effects of Pien Tze Huang on the protection of the nerves, blood vessels, neurons and glia, to explore its protective mechanism and possibly to perform clinical studies.

7.5 Conclusions

(1) Ischemic stroke is a kind of cerebral vascular disease with high disability rate and case fatality rate; hypertension is the main risk factors that may aggravate the prognosis of the disease.

(2) Permanent occlusion of both the left and right common carotid arteries in SHR is a suitable model of chronic brain ischemia combined with hypertension.

(3) Chronic brain ischemia combined with hypertension in the rat hippocampus causes an increased induction of antioxidation activity, and more damage than ischemia alone.

(4) Pien Tze Huang has a protective role in the prevention of brain damages caused by ischemia. In addition, it delays the onset time of stroke and reduce the severity of stroke deficits in SHRsp rats.

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