In vivo and *in vitro* Studies of the Anti-oxidative, Anti-inflammatory and Anti-apoptotic Effects of Gastrodiae Rhizoma Water Extract on Ischemic Stroke

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Abstract of thesis entitled:

Stroke and related cerebrovascular disease is the top second cause of deaths worldwide. Ischemic stroke accounts for about 88% of all strokes. The currently used drugs on stroke treatment aim to prevent subsequent second stroke by anti-coagulation but not the pathological consequence of ischemic stroke including oxidation, inflammation and apoptosis. Gastrodiae Rhizoma, known as Tianma, is a traditional Chinese herbal medicine for treating neural diseases such as paralysis, epilepsy, stroke, etc. We hypothesized that Tianma water extract could be neuroprotective to ischemic stroke. The objective of our study is to investigate the anti-oxidative, anti-inflammatory, and anti-apoptotic effects of Tianma water extract using *in vivo* middle cerebral artery occlusion (MCAo) rat model, as well as *in vitro* H_2O_2-induced oxidative model, oxygen-glucose deprivation (OGD) reperfusion model on rat pheochromocytoma cell line (PC 12), and LPS-induced inflammation on mouse macrophages (RAW 264.7).

In the MCAo model, the effect of Tianma extract on the production of anti-oxidative enzymes and pro-inflammatory cytokines were investigated. The results showed that oral administration of 1 g/Kg of Tianma extract could significantly ameliorate brain infarct volume and the average neurological score by 41% and 30%, respectively, as compared with water-treated control. Tianma treatment also expressed significant upregulation of the anti-oxidative enzymes activities including superoxide dismutase (SOD) and catalase (CAT) in MCAo operated rats. Besides, Tianma extract could significantly suppressed the pro-inflammatory cytokines IL-6 and TNF-α production.

In order to study the underlying mechanisms of the neuroprotective effects of Tianma extract, the production of reactive oxygen species (ROS) was mimicked by

the H_2O_2-induced oxidative model in PC 12 cells. Our results demonstrated that 700 μg/mL of Tianma extract could significantly rescue PC 12 cells survival under 75 μM H_2O_2 from 59.9% to 93.6%. Besides, oxygen-glucose deprivation reperfusion model was employed to mimic the *in vitro* ischemia and reperfusion situation. Tianma extract (1000 μg/mL) could significantly increase the cell viability from 11.5% to 71% and reduce the intracellular ROS level from 99.6% to 71.8% after OGD. In addition, Tianma extract (1000 μg/mL) also exhibited anti-inflammatory effect by inhibiting LPS-induced NO production, pro-cytokines IL-6 and TNF-α production by 40%, 36% and 46%, respectively, as well as attenuating the inflammatory effectors iNOS and COX-2 expression in RAW 264.7 cells. Furthermore, Tianma extract exhibited the anti-apoptotic effect by significantly promoting cell cycle transition after OGD. We also noted that Tianma extract could significantly reduce the cleavage of PARP, a marker for apoptosis and increase the anti-apoptotic Bcl-2/ pro-apoptotic Bax protein expression ratio.

In conclusion, our study provided the first scientific evidence showing the anti-oxidative, anti-inflammatory and anti-apoptotic effects of Tianma water extract, which accounts for the neuroprotection against cerebral ischemia and reperfusion in the MCAo rat model, suggesting its benefits to patients with ischemic stroke.

摘要

中風和相關的腦血管疾病，是全球第二個主要死亡原因，缺血性中風約佔當中的 88%。目前治療中風是依靠溶栓治療以減低日後再次復發的風險，但並非針對缺血性中風病變的結果，包括氧化，炎症和細胞凋亡。天麻是傳統用於治療神經系統疾病，如癱瘓，癲癇和中風等的中藥。我們推測，天麻水提取物(下文簡稱「天麻」)可能對缺血性中風有神經保護作用，以保護缺血後的腦細胞損傷。我們研究的目的是通過體內大鼠大腦中動脈梗塞模型、體外細胞模型包括在大鼠腎上腺髓質嗜鉻瘤分化(PC 12)細胞上進行以 H_2O_2 誘發氧化及「氧糖去除」腦梗塞細胞模型並且以脂多糖在小鼠單核巨噬細胞白血病(RAW 264.7)細胞上誘發炎症，評估天麻的抗氧化，抗發炎和抗凋亡對缺血性中風後的神經保護功效。

大腦中動脈梗塞模型的結果顯示口服 1 克/公斤天麻的大鼠，腦梗塞及神經功能缺損比對照組顯著分別降低 41% 及 30%。另一方面，天麻在體內實驗中顯著提升抗氧化酶的活性；同時，結果顯示天麻亦能有效抑制促炎細胞因數 IL-6 和 TNF-α的產生。

為了研究天麻對神經細胞的保護機理，我們在 PC 12 細胞上以 75 微摩爾 H_2O_2 誘發氧化，模仿腦缺血時所產生的活性氧化物(ROS)。研究結果顯示 700 微克/毫升的天麻能顯著將 PC 12 細胞的存活率從 59.9%提升至 93.6%。在模仿體外缺血灌流損傷情況的「氧糖去除」腦梗塞體外細胞模型中，1000 微克/

毫升天麻能顯著將 PC 12 細胞的存活率從 11.5%提升至 71%，並將「氧糖去除」後細胞內 ROS 水準從 99.6%壓抑到 71.8%。此外，1000 微克/毫升天麻有效抑制 RAW 264.7 細胞因脂多糖誘發炎症中所產生的一氧化氮、促炎細胞因數 IL-6 和 TNF-α分別達 40%，36%和 46%，同時天麻亦能抑制氮合成酶及環氧合酶蛋白表達。除此之外，結果亦顯示出天麻顯著促進「氧糖去除」後細胞週期的過渡，顯著降低 DNA 修復酶—PARP 的裂解，並增加抗凋亡 Bcl-2/促凋亡 Bax 蛋白表現率。以上的結果均反映出天麻具有抗氧化、抗發炎和抗凋亡作用。

總括而言，本研究首次透過科學化研究顯示天麻水提取物具有抗氧化、抗發炎和抗凋亡作用，因而在大鼠大腦中動脈梗塞模型作出神經保護作用，以保護缺血後的腦細胞損傷，此研究或可對缺血性中風患者提供嶄新的治療方向。

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Chapter 1 Introduction

1.1 Cerebral stroke

1.1.1 Epidemiology

Cerebral stroke is one of the cerebrovascular diseases with high incident rate and mortality (Feigin, 2005; Thorvaldsen *et al.*, 1995). According to American Heritage Medical Dictionary, stroke is referred to "A sudden loss of brain function caused by a blockage or rupture of a blood vessel to the brain, resulting in necrosis of brain tissue and characterized by loss of muscular control, diminution or loss of sensation or consciousness, dizziness, slurred speech, or other symptoms that vary with the extent and severity of brain damage." Stroke is also known as cerebral accident, cerebral infarcts or cerebrovascular accident (American Heritage Medical Dictionary, 2000). Common symptoms accompanied stroke include a combination of sudden numbness or weakness of face, arm or leg especially on one side of body (motor symptoms), loss of speech (speech disturbances), altered feeling or loss of vision in specific patterns (sensory symptoms), etc. (Yoon *et al.*, 2001). If these neurologic deficits last less than 24 hours, it is considered as the "Transient ischemic attack" (TIA). TIA is rarely attributed to focal cerebral or retinal ischemia, due to the short duration of inadequate blood supply from either arterial thrombosis or embolism.

For the TIA of more than 24 hours, it is considered as stroke, implying the interruption of blood supply to brain and can be further divided into hemorrhagic stroke or ischemic stroke. For the occlusion of a cerebral artery, it is classified as the ischemic stroke while the rupture of a cerebral artery is termed as the hemorrhagic stroke (Edvinsson and Povlsen, 2011). The neuronal cell death resulted from both hemorrhagic and ischemic stroke due is related to inadequate supply of oxygen and glucose (Macdonald and Stoodley, 1998; Siesjo, 1993; Symon, 1993).

Hemorrhagic stroke can be further divided into parenchymal hemorrhage or subarachnoid hemorrhage depending on the injury site (Bradley, 1993; Broderick *et al.*, 1993). For hemorrhagic stroke, the blood leaked out would accumulate and compress on the surrounding brain tissue. The occurrence of hemorrhagic stroke is closely related to the weakened blood vessels, including aneurysms and arteriovenous malformations (AVMs) (Jane *et al.*, 1985; Redekop *et al.*, 1998). Although the prevalence rate of hemorrhagic stroke accounts for only 8-18% of strokes (Feigin *et al.*, 2003), the mortality rate is much higher than that of ischemic stroke (Morgenstern *et al.*, 2010).

About 87% of the total number of stroke cases is due to ischemic stroke, which is the most common type of stroke (American Heart Association, 2009). The ischemic stroke can be further classified into thrombotic and embolic depending on the original blood clot formed in atherosclerosis (Fisher *et al.*, 2005; Nagai *et al.*, 2001). For the embolic stroke, the emboli refers to migrating blood clots which usually arisen from the heart or large arteries of the upper body and travel to the cerebral arteries via bloodstream. Atrial fibrillation is one of the main causes for embolic stroke (Hara *et al.*, 1995; Meldrum *et al.*, 1996; Petersen and Godtfredsen, 1986). For thrombotic ischemia, the clot is formed by atherosclerosis locally. The risk for thrombus formation and platelet adherence is highly related to arterial stenosis (Kasner *et al.*, 2006; Siebler *et al.*, 1995). Regardless to the embolic or thrombotic stroke, once the artery supplying to the brain undergoes occlusion, it will lead to focal ischemia. In particular, the middle cerebral artery (MCA) is the most commonly occluded vessel in ischemic stroke patients (O'Sullivan *et al.*, 2007). During cardiac arrest, the blood supply to the whole body may be suspended, including the whole brain. Under this situation, one would have global ischemia (Neumar *et al.*, 2008). Severe synaptic and cognitive dysfunctions could result (Jake

T. *et al.*, 2013).

Stroke is a non-communicable disease as well as one of the increasing health burden-causing diseases in the world. According to World Health Organization (WHO), stroke accounts for about 9.7% of the world's total death in 2004 and is also known as the second leading cause of death over the world, which is just less than coronary heart disease (Mathers *et al.*, 2008). Every year, about 16 million of first-ever strokes occur in the world (Di Carlo, 2009). Stroke caused an estimated 5.7 million deaths in 2005. From estimation, the number of global deaths is accordingly projected to rise to 6.5 million in 2015 and to 7.8 million in 2030 (Strong *et al.*, 2007). Stroke is predicted to be one of the four leading causes of death worldwide in 2030 (Mathers *et al.*, 2008). The mortality rates of stroke are obviously higher in non-industrialized countries (Aje and Miller, 2009) which might be related to different lifestyles. The stroke incident rate was the highest in Japan while the lowest rates were among Great Britain, Germany and New Zealand (Feigin *et al.*, 2003).

Almost 50% of stroke patients would be left with physical or cognitive impairment to different extents (Young and Forster, 2007). Poststroke dementia (PSD), which is any kind of dementia occurred after stroke, causes high dependency for stroke survivors after stroke. The onset of PSD in stroke survivors is up to 30% (Leys *et al.*, 2005). These subsequent problems put a great economic burden to stroke patients and their relatives and so as to the society. Stroke is a global health issue. About 85% of all stroke deaths are found in low- and middle-income countries, which accounts for 87% of total losses and is equivalent to 72 million per year worldwide in terms of disability-adjusted life years (DALYs, which is equal to the years of life lost due to mortality + years lived with disability) due to stroke (Lopez *et al.*, 2006). The mean lifetime cost due to ischemic stroke in the United States has been estimated at US$140,048, including rehabilitation, inpatient care, and

necessaries that follow-up (Taylor *et al.*, 1996).

The WHO predicted that stroke and coronary-artery disease together will be the leading cause of lost healthy life-years within 10 years (World Health Organization, 2000b). In Hong Kong, the stroke mortality is annually above 3000 and contributes to 7.4% of total deaths in 2012 (Centre for Health Protection, 2013). Moreover, the stroke-related costs have caused a great public health burden on the local medical system, which account for about 5% of the bed use in Hospital Authority hospitals (Department of Health, 2006)

1.1.2 Risk factors and symptoms

Reported by WHO (McKay *et al.*, 2004), coronary heart diseases and stroke were related to hundreds of risk factors. The factors contributing to these two diseases are quite overlapping. They could be further divided into non-modifiable risks and modifiable risks.

1.1.2.1 *Non-modifiable risks*

Similar to other cardiovascular diseases, the risk of getting stroke is closely related to the age. After the age of 55, the risk of stroke would double every 10 years (McKay *et al.*, 2004). However, a recent report stated that the age onset of stroke has been delayed in many developed countries. The average age of stroke incidence in the study is about age 70, probably related to an increasing age of onset of chronic disease (Shiue, 2011). From the data in 2005, the rate per 1000 of stroke patients of ages 0-59 was only 0.1, while those of ages 60-69 raised to 3.0 and those of ages 70 or above further increased to 12.5 (Strong *et al.*, 2007).

Gender is one of the non-modifiable risks of stroke. Men have higher rates of coronary heart disease than women at premenopausal age (McKay *et al.*, 2004). For

the incidence of stroke and stroke prevalence, men would also have higher than that in women (Appelros *et al.*, 2009; Wyller, 1999). Moreover, the mean age at the first-ever stroke in men was age 68.6, while in women was 72.9 (Appelros *et al.*, 2009). One of the possible explanations would be genetic factors, yet this assumption lacked literature support (Casas *et al.*, 2004). However, a systematic review found that if one had a parental history of stroke; women were more likely than men to have stroke (Touzé and Rothwell, 2008). Another possible reason for the stroke incidence rate between different genders may be related to the positive effects of estrogen acting on the cerebral circulation and decreasing cerebral vascular tone (Krause *et al.*, 2006). Women are exposed to ovarian estrogens throughout lifetime that might protect them against ischemic stroke, especially the non-embolic type (de Leciñana *et al.*, 2007). But this effect seemed diminished after menopause (Murphy *et al.*, 2004) with the loss of body estrogen. The difference of lifestyle between female and male might also be a cause. For instance, female usually had a better knowledge of stroke risk factors and awareness towards stroke than male (Stroebele *et al.*, 2011), etc.

WHO has also reported that people with heredity or family history of stroke would increase the risk of having stroke. The risk would increase if a first-degree relative had the coronary heart disease or stroke before the age of 55 or 65 years for male or female, respectively (McKay *et al.*, 2004). The risk for these people would be doubled in comparison with those without any positive familial history of stroke (Liao *et al.*, 1997).

Children with sickle cell disease (SCD) would have a great chance for having stroke. The occurrence among the SCD children could be as high as 7% to 13%, leading to motor disability, neuropsychological impairment, and death (Ohene-Frempong *et al.*, 1998). The first stroke could occur when the SCD children

were of only 20-month of age (Powars *et al.*, 1978). This was due to the high rate of formation of blood clot among SCD patients. Stroke occurred when this happened in large cerebral vessels, especially in the internal carotid artery (Stockman *et al.*, 1972).

Ethnicity or race may also be one of the non-modifiable risks. The blacks have higher stroke incidence rate, more severe strokes, and higher stroke mortality than whites in US (Gillum, 1988). This may be related to the differences in terms of severity of stroke risk factors, biological differences as well as the lower socioeconomic status (Frey *et al.*, 1998; Gillum, 1999; Sacco *et al.*, 2001). The ages of blacks and Hispanics having strokes were found to be lower than whites. Besides, hypertension associated with stroke in blacks and Hispanics was more prevalent than in whites with stroke. Moreover, the black stroke patients were usually untreated. They hardly visited a physician 1 year after their stroke (Sacco *et al.*, 1995). Blacks and Asians have higher incidence rates in the intracranial occlusive cerebrovascular disease, while whites had more extracranial disease (Feldmann *et al.*, 1990). This might be tied to the different lifestyle and chronic disease epidemiology between whites and blacks. Blacks were more used to become hypertensive, diabetic, or smokers (Inzitari *et al.*, 1990). In addition, the blacks had higher mutation rate in the T594M sodium-channel β subunit which increased sodium-channel activity and thus raising the blood pressure in those with this mutation. This could explain why hypertension, a modifiable risk contributing the most to ischemic stroke (Sacco, 1997; Sacco *et al.*, 1997), was more commonly found in blanks than whites (Baker *et al.*, 1998).

A genetically inherited vascular disease called "Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy" (CADASIL), with missense mutations in the NOTCH3 gene was responsible for the malformation of blood vessels in brain that highly increases the risk of strokes (Chabriat *et al.*, 2009;

Tournier-Lasserve *et al.*, 1991). The symptoms of CADASIL, such as stroke, usually surfaced around the age of 45 (Yao *et al.*, 2012).

1.1.2.2 Modifiable risks

Modifiable risks for stroke means that they can be treated or controlled. The modification of these risks can result in significant reductions in the occurrence of stroke and its mortality. These risks are usually referred to the underlying pathophysiology, as well as lifestyle-related factors (Sacco, 1997).

Hypertension is one of the modifiable risks contributing the most to ischemic stroke (Sacco, 1997; Sacco *et al.*, 1997). More than 50% of strokes can be explained by hypertension in both gender and all age groups (Lawes *et al.*, 2008). The WHO report for 2012 stated that about 51% of stroke is due to hypertension (World Health Organization, 2012). A literature has reviewed that in a randomized controlled trial for the patients with mean age of 70 years, a reduction of 10 mm Hg in systolic blood pressure (BP) correlated with about 30% reduction in risk of stroke. This claimed that the stability of normal BP is vital for stroke prevention (Lawes *et al.*, 2004; MacMahon *et al.*, 1990). However, the impact of hypertension may reduce with the increased age (Sacco *et al.*, 1997; Whisnant, 1996). People with a blood pressure reduction within the normotensive range according to their own ages would have a reduction of 40% and 38% for fatal stroke and all stroke, respectively (Lawes *et al.*, 2004; Sacco *et al.*, 1997). As hypertension is a common risk factor for both hemorrhagic and ischemic strokes (Mohr *et al.*, 1997; Sacco *et al.*, 1997), the treatment of hypertension could reduce the occurrence of both strokes (Lawes *et al.*, 2004). The drugs treating hypertension have been proven to have stroke prevention effects. Renin angiotensin system (RAS) was closely related to stroke (Iadecola and Gorelick, 2004; Schrader *et al.*, 2007). RAS blocker such as Ramipril could reduce all

stroke and fatal stroke by 32% and 61%, respectively when compared with placebo (Bosch *et al.*, 2002). A study also found that another anti-hypertensive drug, Losartan, could significantly reduce the rate of fatal and non-fatal stroke by 25% among the enrolled hypertensive patients with left ventricular hypertrophy (Dahlöf *et al.*, 2002).

Another vital factor that increases stroke risk is hyperlipidemia (McKay *et al.*, 2004; Meyer *et al.*, 1987), meaning the abnormal elevation of total cholesterol, low-density lipoprotein (LDL) and triglyceride in blood (Dorland, 2007). A commonly used drug for the management of hyperlipidemia, Pravastatin, when compared with placebo treatment, showed that besides the significant reduction in LDL cholesterol and total cholesterol, it was also associated with about 30% lower incidence of stroke (Farnier and Davignon, 1998; Sacks *et al.*, 1996).

Commonly, people may believe that the major risk of smoking is respiratory related diseases, but the truth is that smokers develop cardiovascular diseases as well, including stroke (McKay *et al.*, 2004). Smoking could almost double the risk of stroke (Shinton and Beevers, 1989), while no relationship with the amount of daily cigarettes smoked and the onset of stroke was reported (Håheim *et al.*, 1993). The cigarette smoking had been proven to be synergistic in the development of stroke (Schwartz *et al.*, 2004). For a 26 year-follow-up study of 4255 smokers, the average annual incidence of stroke was 0.415% and the incidence rate in men was about 20% higher than that in women (Wolf *et al.*, 1988). But the risk would decline after cessation of smoking and return to normal after 5 years (Kawachi *et al.*, 1993).

Light or moderate alcohol use has been proposed to have a protective effect on ischemic stroke prevention (Gill *et al.*, 1986; Mukamal *et al.*, 2005). Moderate alcohol consumptions suggested by US government for men and women were 5-30 g per day and 5-15 g per day, respectively (Peterkin, 1990). However, heavy alcohol consumption would induce hypertension and cerebral blood flow reductions

(Gorelick, 1989), thus increasing the risk of having stroke.

The lifestyle, such as fair amount of physical activities, balanced diet and body mass index (BMI), plays an important role in preventing stroke or other cardiovascular diseases (Chiuve *et al.*, 2008). Moderate and high levels of physical activity could certainly reduce the risk of stroke (Do Lee *et al.*, 2003). The protective effect of physical activity towards stroke was independent to age, gender, race and ethnic groups (Abbott *et al.*, 1994b; Do Lee *et al.*, 2003; Goldstein *et al.*, 2006; Sacco *et al.*, 1998). However, the amount of exercise may be inversely proportional to the risk of hemorrhagic stroke in some studies (Hu *et al.*, 2000; Lee *et al.*, 1999a).

There is no doubt that the higher the BMI, the higher the risk of ischemic stroke. The risk of stroke for obese individual (BMI $\geq$ 25) (World Health Organization, 2000a) was doubled when compared with normal individual (Abbott *et al.*, 1994a). The risks of stroke for male and female were 195% and 172% higher, respectively (Kurth *et al.*, 2002; Kurth *et al.*, 2005). A diet also associated with the incidence of stroke (Waxman and World Health Assembly, 2004). For example, daily consumption of fruit and vegetables thrice could reduce the risk by about 30% when compared with those who has less than once daily intake (Bazzano *et al.*, 2002). High fat diet would nonetheless increase the risk of stroke (O'Donnell *et al.*, 2010) as well as lead to the development of hypertension and hyperlipidemia (Boden-Albala & Sacco, 2000). High salt diet would significantly increase stroke risk (Nagata *et al.*, 2004) as well as the incidence of hypertension (Appel *et al.*, 2006). People with Vitamin B_6, B_{12} and C deficiency would have higher chance of stroke (Kelly *et al.*, 2003; Kurl *et al.*, 2002; Weikert *et al.*, 2007).

Diabetes mellitus (DM) patients have relative high ischemic stroke risk, up to almost 6-fold (US Preventive Services Task Force, 1989). The high blood glucose accounted for about 16% of death of stroke in 2012, as indicated by WHO (World

Health Organization, 2012). It is believed that diabetic patients would be more likely to have hypertension (Abbott *et al.*, 1987; Kissela *et al.*, 2005) and therefore higher risk for stroke. The risk may also associate with the high blood glucose level among diabetes. Every 1 mmol/l lower of fasting glucose could lower the risk of stroke by 21% (Danaei *et al.*, 2006; Studies, 2004) and the treatment for DM patients may lower the risk of stroke. For DM Type I patients receiving anti-glycaemia intensive treatment, the combined risk of non-fatal MI, stroke, or death from cardiovascular events was reduced by 57% (Nathan *et al.*, 2005). For DM Type II patients, an antiplatelet drug such as aspirin could lower the risk of ischaemic stroke by 30%, but with increment of risk of hemorrhagic stroke by 22% (Trialists'Collaboration, 2002). Similar effect was found in using angiotensin II receptor blocker (ARB), but this might be due to the anti-hypertension effects (Wachtell *et al.*, 2005).

There has been a strong association of migraine and ischemic stroke. The risk of ischemic stroke was almost doubled in people with migraine (Buring *et al.*, 1995; Etminan *et al.*, 2005), and further heightened for those using oral contraceptives and were heavy smokers (Etminan *et al.*, 2005; Tzourio *et al.*, 1995).

1.1.2.3 Symptoms and diagnosis

Due to different positions and severities of brain infarct occurred among stroke patients, the symptoms of stroke are consisted of many variations. Common symptoms for stroke examination include sudden facial paresis, aphasia, hemiparesis, hemisensory deficit, etc. (Brott *et al.*, 1989; Cote *et al.*, 1989; Gelmers *et al.*, 1988; Shinar *et al.*, 1985). However, for the diagnosis of ischemic or hemorrhagic stroke, neuroimaging such as computerized tomography (CT) scanning is crucial for the differentiation (Muir *et al.*, 1996). Los Angeles Paramedic Stroke Scale (LAPSS), Cincinnati Prehospital Stroke Scale (CPSS), and Face Arm Speech Test (FAST) are

another three commonly used rapid ambulance protocols to assess patients with suspected acute strokes, (Harbison *et al.*, 1999; Harbison *et al.*, 2003; Hurwitz *et al.*, 2005; Kidwell *et al.*, 1998). The FAST is used for the assessment of stroke by checking the facial asymmetry, speech disturbance and arm weakness, especially those with TIA (Holzheimer *et al.*, 2001).

The middle cerebral artery (MCA), the largest branch of the internal carotid, is one of the most commonly affected arteries in human strokes (Bousser *et al.*, 1992; Rordorf *et al.*, 1998). Loss of consciousness may occur after MCA occlusion and result in seizures in most cases (Bousser *et al.*, 1981). It also occurs in hemorrhagic strokes, but is usually accompanied with headache and vomiting (Caplan, 2009). Motor deficits, mainly partial hemiparesis, may occur in MCA occlusion as well. The National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) data bank project from US has concluded that the hemiparesis was commonly observed in hip, foot, shoulder, and hand for about 70% of cases (Mohr *et al.*, 1984). Since the MCA supplies a part of the frontal lobe and the lateral surface of the temporal and parietal lobes (Standring *et al.*, 2005) where the primary motor and sensory areas, the areas for speech and the superior portion of the optic radiation (Nowak *et al.*, 2008; Schiemanck *et al.*, 2008) are all located here, therefore besides hemiparesis, visual deficit, like hemianopia and autonomic dysfunction such as excessive sweating (hyperhidrosis), are common manifestations (Labar *et al.*, 1988). Aphasia and apraxia are the most performed manifestations of hemispheric lesion of MCA occlusion (Goodglass and Quadfasel, 1954; Tognola and Vignolo, 1980). Aphasia includes difficulties in producing words or sounds, impaired expression of either spoken or written language and often missing key words, etc. (Benson *et al.*, 1973; Mohr *et al.*, 1978). Apraxia refers to the inability to perform a previously learned task when the action is impaired but not lost. It is also known as “dyspraxia”

(Mohr *et al.*, 1978; Mohr *et al.*, 1984).

The anterior cerebral artery (ACA) is responsible for the blood supply covering the whole medial surfaces of the frontal and parietal lobes, the frontobasal cerebral cortex, the anterior diencephalon (Standring *et al.*, 2005). The ACA occlusion is rare and accounted for 2% of cases (Baird, 2008). The posterior cerebral artery (PCA) lesion is uncommon but fatal (Martin-Schild, 2013). Hemiparesis of leg and occasional aphasia may be related to the occlusion of the left ACA (Benson *et al.*, 1973; Mohr *et al.*, 1978; Roach *et al.*, 2010). The most common consequences of PCA occlusion are visual and sensory deficits (Fisher, 1986).

1.1.3 Mechanisms of cell injury

1.1.3.1 Energy failure and loss of ionic homeostasis

Like all cells in human bodies, the nerve cells required continuous supply of oxygen and glucose for the maintenance of the high aerobic metabolic rate and to release energy to the tissue. The cerebral blood flow (CBF) was normally 50-60 mL/100 g/mins, which was typically 15% of the total resting cardiac output. Unconsciousness would be caused by cessation of CBF within 5-10 seconds due to deprivation of oxygen and glucose to nerve cells. Both cellular alternation and cell death would occur if the CBF was less than 8ml/100g/min (Edvinsson *et al.*, 1993). During Ischemic stroke, the CBF in the severely ischemic core would be $\leq$ 6 mL/100 g/mins (Kaufmann *et al.*, 1999).

The glycogen storage in brain was low during cerebral ischemia. Though anaerobic glycolysis may be initiated, the energy produced was not enough for the survival of nerve cells. The ATP-driven transmembrane ion pumps, especially Na^+/K^+-ATPase for the maintenance of ion concentration gradient would fail. Under normal situation, high intracellular potassium (K^+) ion concentration as well as the

low intracellular sodium (Na^+) ion concentration was maintained. However, the failure of Na^+/K^+-ATPase would lead to efflux of K^+ from cells and Na^+ flow into the cell, thus causing the influx of Calcium (Ca^{2+}) ion through voltage-sensitive Ca^{2+} channels and influx of chloride (Cl^-) ion and water. Cell swelling and finally edema occurred. The elevated intracellular Ca^{2+} also led to the release of neurotransmitters such as glutamate (Brady *et al.*, 2005). Moreover, when anaerobic respiration occurred in nerve cells, by-products especially lactate would be produced. This caused tissue acidosis (Paschen *et al.*, 1992). The intracellular pH value was reduced as a result. Thus the buffering capacity of bicarbonate was reduced and caused further tissue acidification (Macdonald and Stoodley, 1998; Zauner *et al.*, 2002). With the accumulation of intracellular H^+, Na^+, and reduced intracellular K^+, all would inhibit the glutamate re-uptake. The elevated extracellular glutamate concentrations would induce severe excitotoxic damage to target neurons, acting just like a neurotoxin (Choi, 1992). In summary, the shortage of oxygen and substrate as well as the accumulation of metabolic waste are the major causes of ischemic insult (Auer and Siesjo, 1988).

1.1.3.2 Excitotoxicity and calcium-modulated cell damage

Excitotoxicity was regarded as high level of glutamate mediated by Ca^{2+} influx leading to neuronal cell death (Olney, 1969). Glutamate is one of the dicarboxylic amino acids synthesized from 2-oxoglutarate which strongly affects the neurons. It is crucial in the opening of Na^+ and K^+ channels and resulted in rapid excitatory response in most neurons. Besides, it was associated with the slow developing neuroplasticity during learning, memory and neuronal development (Brady *et al.*, 2005; Gibbs and Hertz, 2005). Glutamate is controlled by Ca^{2+}-dependent channel and under normal situation, the synthesis and degradation of

glutamate were performed by enzymes in neuronal cells and glial cells to prevent accumulation of glutamate. The astrocytes also removed extracellular glutamate through glutamate transporters for protection of neurons. Excessive extracellular glutamate was toxic to neurons and glial cells via oxidative stress produced by reactive oxygen species (ROS) formation which was probably the result of glutamate receptor activation (Olney *et al.*, 1979) in mechanisms of neuroinflammation (Farooqui *et al.*, 2008), which finally led to apoptosis of these cells.

Glutamate could bind with numerous ionotropic amino acid receptors, N-Methyl-D-aspartic acid (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate (KA, a glutamate analogue), receptors, as well as metabotropic receptors such as *trans*-1-amino-cyclopentyl-1, 3-dicarboxylate (*trans*-APCD) (Mayer and Westbrook, 1987). During ischemia, the extracellular concentration of glutamate might increase to 1000-fold of normal and resulted in neuronal cell death (Dugan and Choi, 1999). Once glutamate bound to NMDA receptors, the opening of the ion channel was initiated. The stimulated NMDA receptors would be desensitized and become more permeable to Ca^{2+} ions (Mayer and Westbrook, 1987; Monaghan *et al.*, 1989). For the overstimulated AMPA/KA receptors, Na^{+} ions influx occurred resulting in further depolarization and eventually excessive influx of Ca^{2+} ions through voltage-dependent Ca^{2+} ions channel, as the intracellular concentration of Na^{+} was too high and that the Na^{+}/Ca^{2+} ion exchangers were reversed to facilitate pumping in of more Ca^{2+} ions (Yu and Choi, 1997). Both of these would intensify the cytotoxicity caused by increased Ca^{2+} ions concentration.

Numerous lipolytic and proteolytic enzymes, such as diacylglycerol lipase, phospholipases, proteinases and endonucleases, etc. could be activated by the high intracellular Ca^{2+} ions (Choi, 1995). As a result, the cell membrane, cytoskeleton,

DNA would be broken down. Moreover, protein phosphorylation was affected since the activities of protein kinases and phosphatases were altered. NMDA and AMPA/KA receptors were further stimulated leading to the opening of voltage-gated Ca^{2+} channels. The influx of Ca^{2+} was further intensified. This would predispose the activation of enzymatic production of ROS and nitric oxide (NO) (Price *et al.*, 1991; Tymianski and Tator, 1996). NO is generated by neuronal nitric oxide synthase (nNOS) (Bredt *et al.*, 1991). The NO would react with superoxide (one of the ROS) to give rise to a highly reactive free radical peroxynitrite anion ($ONOO^-$) (Ischiropoulos *et al.*, 1992). Lipid peroxidation would occur under the presence of $ONOO^-$ (Brookes *et al.*, 1998), thus resulted in cell membrane disruption. Besides, the phospholipase A_2 (PLA_2) would be upregulated under a Ca^{2+} ions rich environment. PLA_2 was responsible for the cleavage of phospholipids to arachidonic acids, which would be further converted to eicosanoids by free radicals produced in the upstream reaction (Bazan, 1999).

1.1.3.3 Oxidative stress

Normally, about 1-2% of consumed oxygen is converted to ROS, including superoxide, singlet oxygen, hydrogen peroxide and hydroxyl radicals in the mitochondria (Emerit *et al.*, 2004). The amount of ROS will be elevated during ischemia and reperfusion. When acute stroke damage occurred, the localized hypoxia was resulted (Choi, 1990) and cerebral infarct developed. CBF fell below 20 mL/100g per min contributed to the tissue necrosis and neuronal cell death, due to abnormalities of neurochemicals accompanied with the reduction of CBF (Pulsinelli, 1992). One of the most vital roles played in the oxidative stress damage during ischemia and reperfusion is the formation of oxygen free radicals (Özben, 1998). When the lone electron of a free radical encountered other molecules, another free

radical would be produced. A chain reaction was initiated and terminated only either by the random collision of two free radicals to form a molecule with a stable bond (Schmidley, 1990).

The free radicals were mainly found in mitochondria where the electron transport chain (ETC) functioned. During ischemia, the limited oxygen supply would suspend the ETC normal function if the inner mitochondrial membrane and resulted in excessive free radicals leakage associated with the ATP generation along the ETC (Traystman *et al.*, 1991), where the normal mitochondria oxygen usage leakage was only about 3% (Dugan & Choi, 1999). It was commonly agreed that most free radicals were generated during reperfusion, the stage which the occluded vessels regained blood flowing again. At this time, the anti-oxidative defence mechanisms of the brain were not yet functional after ischemia and the free radicals worsen the situation during the reperfusion stage (Cao *et al.*, 1988; Ikeda and Long, 1990; Traystman *et al.*, 1991; Yu *et al.*, 2008a).

Molecules with weak bonds, such as sulphur-containing amino acids and the polyunsaturated fatty acids were particularly vulnerable to the free radicals. Due to the high abundance of polyunsaturated fatty acids in central nervous system (CNS), polyunsaturated fatty acids with several double bonds per molecule were quite susceptible to free radical damage (Halliwell and Gutteridge, 1985). Besides area rich with polyunsaturated fatty acids (e.g. membrane lipids), iron rich area (e.g. globus pallidus and substantia nigra of human brain) as well as area with high ascorbic acid (i.e. grey matter and white matter in CNS) were more liable to free radicals damage. Various cell components including cell membranes, mitochondria and DNA could be damaged and resulted in cell death (Özben, 1998).

As mentioned in 1.1.3.2, intracellular Ca^{2+} and Na^{+} concentrations would be elevated during ischemia and result in the disturbance of the mitochondrial electron

transport and led to inhibition of the NADH, the first enzyme in the ETC, followed by release of free radicals, such as ubisemiquinone. The ubisemiquinone would further react with oxygen to form superoxides (Özben, 1998; Traystman *et al.*, 1991). Moreover, mitochondrial permeability transition pores would open under high Ca^{2+} concentration, causing the swelling of mitochondria and damage of mitochondrial membrane leading to further release of free radicals (Warner *et al.*, 2004). The increased Ca^{2+} concentration would activate the nitric oxide synthase (NOS) to produce NO. The accumulated superoxide would react with NO and formed a highly reactive peroxynitrite radical (Özben, 1998). The peroxynitrite would target on lipids and resulted in lipid peroxidation. Cell membrane was thus damaged. In addition, DNA base pairs were also oxidized and caused DNA damage (Giovannelli *et al.*, 2002). The poly (ADP-ribose) polymerase (PARP) for DNA repair was thus stimulated (Warner *et al.*, 2004) and there would be further depletion of cellular ATP for producing the ADP-ribose polymer. Apart from DNA damage, the tyrosine in proteins was nitrosylated by peroxynitrite to form nitrotyrosine. Under nitrosylation, numerous anti-oxidative enzymes such as Cu/Zn-superoxide dismutase, Mn-superoxide dismutase and cytochrome c oxidase (mitochondrial complex IV) were inactivated (Dugan and Choi, 1999; Mehta *et al.*, 2007). Hence the anti-oxidative ability of the cells was reduced and mitochondrial dysfunction resulted.

Under high Ca^{2+} concentration environment, the PLA_2 would be upregulated, promoting the cleavage of phospholipids to arachidonic acids, which would be further converted to eicosanoids, which are lipid signalling molecules including prostaglandins and thromboxane A_2 (Bazan, 1999). This conversion was catalyzed by cyclooxygenases, lipooxygenases and the presence of superoxide. Large amount of fatty acids would be released (Gaudet and Levine, 1979; Gaudet *et al.*, 1980; Shohami

et al., 1982). When free radical attacked the lipid, peroxyl radicals and lipid radicals were then formed and started to attack other lipid molecules, just like a chain reaction and cause cellular membrane damage (Cherubini *et al.*, 2005; Polidori *et al.*, 1998; Watson *et al.*, 1984; Yoshida *et al.*, 1982). Free radicals would trigger both cytotoxic (first stage) and vasogenic edema (later stage) as vascular permeability increased due to abnormal ions concentration. The blood–brain barrier (BBB) was still intact during cytotoxic edema (Özben, 1998) but uncontrolled fluid leakage from the blood to the brain parenchyma eventually occurred after ischemia through a weakened BBB and vasogenic edema became evident. The net volume of the brain increased and eventually led to organ failure (Heo *et al.*, 2005).

The glutamate reuptake would be inhibited by the free radical due to oxidation of the glutamate transporter 1, excitatory amino acid transporter (EAAT)-2 and EAAT-3, glutamate excitotoxicity was further intensified (Camacho and Massieu, 2006; Trotti *et al.*, 1997).

1.1.3.4 Inflammation

Inflammation occurred a few days after ischemia. Inflammatory cells and mediators such as cytokines, endothelial cells, leukocyte adhesion molecules, NO and cyclooxygenase (COX-2) all contributed a lot to ischemic brain injury (Wood and Rothwell, 1998).

Firstly, due to the presence of leukocytes and the numerous damages induced, the vascular capillaries become obstructed, thus reduced local tissue perfusion (Ames, 1968; Del Zoppo *et al.*, 1991). The prothrombotic action of leukocytes was initiated causing the aggregation of platelets and leukocytes in stroke patients. Leukocytes were able to produce plasminogen-activating factors and elastase (Grau *et al.*, 1994a; Grau *et al.*, 1994b), and caused further occlusion of the cerebral arteries or even

secondary ischemia. Moreover, the leukocytes were closely related to the production of free radicals and enzymes such as NADPH oxidase and iNOS (Wood and Rothwell, 1998). NADPH oxidase has been proven to damage the neutrophils, which played an important role in reducing free-radical formation and thus reducing the brain infarct (Walder *et al.*, 1997). iNOS was responsible for the production of NO and was further converted into $ONOO^-$ (Özben, 1998).

Intercellular adhesion molecules (ICAMs) played an important role for the ischemia-induced leukocytes accumulation and infiltration, enhancing the leukocyte binding to β_2-integrin receptors and selectins on leukocytes (Wood and Rothwell, 1998). Selectins, integrins and immunoglobulin supergene family adhesion molecules were upregulated after ischemia (Kim, 1996; Zoppo *et al.*, 2000a). Selectin molecules such as E-, L- and P-selectins were responsible for the low-affinity binding among platelets, leukocytes, endothelial cells, neutrophils and monocytes then facilitated the rolling of leukocytes and local inflammation (Wood and Rothwell, 1998). The β_2-integrin and ICAM-1 were necessary for the aggregation and transmigration of leukocytes across endothelium. CD11b/CD18 and CD11a/CD18 molecules, belonging to the integrin family, were expressed in leukocytes and bound to the endothelium counter-receptors of ICAM-1 and ICAM-2, which were of the immunoglobulin supergene family (Kishimoto and Rothlein, 1994). Besides ICAMs, the vascular cell adhesion molecules (VCAMs) are upregulated as well. VCAM-1 would bind to another integrin, $\alpha_4\beta_1$-intergrin to recruit lymphocytes and monocytes to the infarct (Feuerstein, 1997). The recruitment of leukocytes may enhance the inflammation damage. Neutrophils were modified from the predominant leukocytes at the inflammation site, after the infiltration of mononuclear phagocytes (Garcia and Kamijyo, 1974). Matrix metalloproteinase (MMP)-9, which was harmful to the intact blood-brain barrier, was released by neutrophils (Gasche *et al.*, 1999; Gidday *et al.*,

2005).

Two pro-inflammatory cytokines, Interleukin (IL)-1β and tumor necrosis factor (TNF)-α were playing vital roles in stroke (Lipton, 1999). After ischemia, both IL-1β and TNF-α were upregulated by elevated production and release of cytokines from macrophages (Wood and Rothwell, 1998). The increased synthesis and release could be induced by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), free radicals and mitogen-activated protein kinases (Meldrum *et al.*, 1998). Moreover, the activation of NMDA receptor and increase in intracellular Ca^{2+} concentration were believed to be involved in the induction of IL-1β and TNF-α in a more distal manner (Bertorelli *et al.*, 1998). These cytokines worked closely in the upregulation of different adhesion molecules, including E-selectin, ICAM-1, ICAM-2 and VCAM-1. For example, both IL-1β and TNF-α could induce the expression of ICAM-1 (Wood and Rothwell, 1998). TNF-α, on the other hand, was involved in many functions, such as in acute-phase protein secretion and adjustment of vessel permeability (Tracey and Cerami, 1993). IL-1β was biologically active once cleaved by capase-1 and were localized in cerebral vessels, macrophages, etc. after ischemia (Davies *et al.*, 1999; Tilg *et al.*, 1994).

However, these cytokines all might exacerbate the cell injury. For example, IL-1β would stimulate the proliferation of astrocytes (Giulian and Lachman, 1985) and caused cerebral edema (Gordon *et al.*, 1990). IL-1 would induce arachidonic acid production, promotion of NMDA-mediated excitotoxicity and stimulation of NOS (Huang *et al.*, 1994b). IL-6 upregulation was also linked with increased infarction (Tarkowski *et al.*, 1995). TNF-α would increase the permeability of blood-brain barrier and led to direct toxicities to capillaries (Beutler and Cerami, 1987; Goldblum and Sun, 1990).

1.1.3.5 Apoptosis

Apoptosis is known as "the programmed cell death". During ischemic stroke incident, the brain tissue that is exposed to the reduction of blood flow is injured severely and undergoes necrotic cell death. The less severely affected tissue surrounding the core still remained metabolically active (Ginsberg, 1997) and this was known as "ischemic penumbra", the region which provided opportunities for neuronal cells rescue and reduce neuronal cell death (Broughton *et al.*, 2009). Apoptosis is known to be initiated in ischemia-reperfusion. There are two general pathways for mediation of apoptosis, the intrinsic and extrinsic pathways (Broughton *et al.*, 2009).

The intrinsic pathway is mitochondrial-dependent, involving the interactions and regulations of proapoptotic protein Bax and other Bcl-2 family members (Lopez-Neblina *et al.*, 2005). One of the most important factors for the intrinsic pathway is the post-ischemic cytotoxic accumulation of intracellular Ca^{2+} ions through the glutamate stimulation on NMDA or AMPA receptors (Simard *et al.*, 2007). The elevated Ca^{2+} ions inside mitochondria would activate calpains and in turn stimulate the proapoptotic protein activation via extrinsic pathway, such as the cleavage of Bcl-2 interacting domain (BID) into truncated active form (tBID) (Culmsee *et al.*, 2005; Love, 2003). BID plays an important role in mediating ischemic neuronal cell death and that BID cleavage and BID gene expression are closely related to ischemic infarct size (Plesnila *et al.*, 2002). tBID would induce conformational changes in other proapoptotic proteins, such as Bak, Bax, Bad, and Bcl-XS (Sugawara *et al.*, 2004) as well as involved with heterodimerization of antiapoptotic proteins, Bcl-2 or Bcl-XL by the BH3 domains (Webster *et al.*, 2006).

The opening of mitochondrial transition pores would cause the release of proapoptotic proteins from the intermembrane space into the cytosol, including

cytochrome *c*, Smac/DIABLO and the serine protease HtrA2/Omi which would lead to the activation of the caspase-dependent mitochondrial pathway (Elmore, 2007). An apoptosome was formed once ATP and cytochrome *c* bound and activated the cytosolic protein Apaf-1 and procaspase-9 (Love, 2003) which was followed by the activation of caspase-9, an initiator of the cytochrome *c*-dependent caspase cascade, then further activated caspase-3 (Sugawara *et al.*, 2004).

Both caspase-9 and caspase-3 were vital in ischemic neuronal death (Namura *et al.*, 1998; Sugawara *et al.*, 2002). Upregulation of caspase-3 and caspase-9 were observed in the animal ischemic model. For caspase-3, it was known as the key mediator of apoptosis in animal models of ischemic stroke. The finding of caspase-3 was similar in ischemic human brain tissue (Rami *et al.*, 2003) in the mediation of apoptosis because it was involved in numerous substrate protein cleavage, such as PARP (Endres *et al.*, 1997; Namura *et al.*, 1998) which would be inactivated after cleaved by caspase-3, leading to DNA injury and apoptotic cell death. However, excessive activation of PARP would result in nicotinamide-adenine dinucleotide and ATP depletion which were tied to cellular energy failure and necrotic cell death (Sugawara *et al.*, 2004). Therefore the genetically deletion or pharmacological inactivation of PARP could result in reduction of cerebral infarct volume after MCAo (Eliasson *et al.*, 1997; Endres *et al.*, 1997).

Inflammatory cytokines, such as IL-1β, TNF-α and transforming growth factor (TGF)-β, derived from macrophages, microglia, glial cells, were observed after ischemia (Brady *et al.*, 2005) and also closely related to the extrinsic apoptotic pathway (Broughton *et al.*, 2009; Cho and Toledo-Pereyra, 2008; Doyle *et al.*, 2008). Apoptotic neuronal cell death could be triggered by these cytokines (Licinio, 1997). For instance, the binding of TNF-α onto Fas receptor could initiate the extrinsic pathway (Bermpohl *et al.*, 2007; Jin *et al.*, 2010; Mehta *et al.*, 2007). TNF receptor

was one of the cell surface death receptors, which also included TNFR-1, $p75^{NTR}$ and Fas (Broughton *et al.*, 2009; Poh Loh *et al.*, 2006). Once activated, the extrinsic receptor pathway of caspase cascades would be switched on (Sugawara *et al.*, 2004). Finally, activation of caspase-activated deoxyribonuclease (CAD) and DNA fragmentation were initiated (Lopez-Neblina *et al.*, 2005).

1.1.4 Current treatment of ischemia

As large amount of neuronal cells in the penumbra area of the brain infarct would die during ischemia via different mechanisms, the treatment of ischemia was focusing on perfusion (restoring blood circulation to affected area) and reduction of neuronal cell death (neuroprotection) (Ferriero, 2004; Moskowitz *et al.*, 2010).

Restoring of blood flow to affected area is the management for acute stroke patients. The thrombolytic drug, intravenous recombinant tissue-type plasminogen activator (rt-PA), is the drug for stroke treatment which was approved by United States Food and Drug Administration (USFDA) in June, 1996 (Adams *et al.*, 1996; Adams *et al.*, 2003) after the report from National Institute of Neurological Disorders and Stroke (NINDS) rt-PA Stroke Study Group established that the rt-PA was beneficial for patients within 3 hours once acute ischemic stroke occurred (NINDS rt-PA Stroke Study Group, 1995). According to Miller-Keane encyclopedia & dictionary of medicine, nursing, & allied health, "Tissue plasminogen activator (tPA) is a serine endopeptidase synthesized by endothelial cells, acting as a physiologic activator of plasminogen catalyzing the conversion of plasminogen to plasmin by hydrolysis of a specific arginine-valine bond when bound to fibrin clots.

The synthesized tPA was known as rt-PA produced by recombinant technology for use in thrombolytic therapy for dissolving blood clots (Brown, 1992). The most well-known recombinant fibrin-specific plasminogen activator of rt-PA is

Tenecteplase (TNK), consisting of alteplase, reteplase and tenecteplase and derived from natural tPA which can lyse thrombus and restore blood flow. The effect of rt-PA was so significant that about 30% of rt-PA treated patients had minimal or no disability at three months after treatments (Tanswell *et al.*, 2002). However, the risk of having intracerebral hemorrhage within 36 hours after the stroke incidence was increased by about 10-fold in rt-PA treated patients (NINDS rt-PA Stroke Study Group, 1995). The time-window for rt-PA treatment was 3 to 4.5 hours after ischemia stroke onset (Hacke *et al.*, 2008; Lansberg *et al.*, 2009). Anticoagulation drugs such as unfractionated heparin (UFH), or simply called heparin and aspirin could be used in anticoagulant therapy (Berge *et al.*, 2000; Kalafut *et al.*, 2000). However, the risk of hemorrhage might increase as well. The use of heparin and aspirin for acute ischemia treatment and secondary stroke prevention had long been a safety issue (Gubitz *et al.*, 2008; Petty *et al.*, 1999). The latest drug approved by USFDA in December, 2012 was Eliquis (apixaban) for reduction of the risk of stroke and systemic embolism in patients with atrial fibrillation. Patients on Eliquis in the clinical trials had lower risks of strokes than those taking warfarin (Hossain and Tanvir, 2013; Rukovets, 2013).

For patients with high risk of intracerebral hemorrhage who were ineligible for rt-PA, stroke thrombus could be treated mechanically by Mechanical Embolus Removal in Cerebral Ischemia (MERCI), approved by USFDA in 2004 (Katz and Gobin, 2006), used direct delivery (during angiography) of a clot-disrupting or retrieval device (only 2-5mm) through catheters during angiography to re-canalize cerebral arteries in acute stroke (Berlis *et al.*, 2004; Furlan, 2003). However, the mortality is still high, due to limited use of MERCI (Smith *et al.*, 2005).

Treatments for neuroprotection can work on different pathways as mentioned in Chapter 1.1.3. Nicardipine, dihydropyridine derivatives and nimodipine, etc. were

commonly used calcium channel blockers (CCBs) (Inzitari and Poggesi, 2005) for treating hypertension and have been proven to reduce the risk of stroke (Angeli *et al.*, 2004; Verdecchia *et al.*, 2005). Drugs function with glutamate inhibition or GABA stimulation could be a possible treatment. GV150526 and Clomethiazole were glycine-site (NMDA-receptor) antagonist and enhancer of GABA-receptor, respectively (De Keyser *et al.*, 1999). Both of them entered the clinical trials (Devuyst and Bogousslavsky, 2010; Martinez-Vila and Sieira, 2010). Scientists have performed many researches on neuroprotection through other pathways such as anti-oxidation, anti-inflammation and anti-apoptosis. Some of the researches might even enter clinical trials (De Keyser *et al.*, 1999; Ikonomidou and Turski, 2002; Yamaguchi *et al.*, 1998). However, none of these have gained USFDA approval as an acute ischemic stroke treatment yet (Roach *et al.*, 2010).

1.2 Chinese herbal medicine

1.2.1 Traditional Chinese medicine theory on stroke

Traditional Chinese medicine (TCM) has been used in China for thousands of years and the therapeutic effects of TCM on stroke have been well documented in numerous Chinese literatures (Zhou and Xiao, 1997).

TCM emphasized the relationship between human and the nature. Any imbalance would cause changes in physiological function and even gave rise to diseases (Maciocia, 2005). Stroke was known as "*bao jue*" (「*薄厥*」), "*da jue*" (「*大厥*」), etc. titled *Su Wen* (Simple Questions) in *Huang Di Nei Jing* (The Yellow Emperor's Inner Classic),「*陽氣者，大怒則形氣絕而血菀於上，使人**薄厥**，有傷於筋，縱其若不容。*」 and 「*血之與氣，並走於上，則為**大厥**，厥則暴死。氣復反則生，不反則死。*」(Veith, 2002). In TCM point of view, stroke was a syndrome called "wind stroke". Patients with wind stroke were characterized with "facial paralysis, dysphasia, or aphasia and/or hemiplegia". According to TCM theory, wind stroke is caused by the disturbance of "internal wind" and "external pathogenic wind". The interactions with other elements might lead to Yin or Qi weakness and accorded disturbance of the blood and Qi circulation and deficiency of 'liver-Yin' and 'kidney-Yin'. Finally these would cause the incidence of wind stroke which could be further classified according to the symptoms to "viscera stroke" (with loss of consciousness) and "channel-collateral stroke" (without loss of consciousness) (Gong and Sucher, 1999). The damage to the brain during many "strokes" was caused by excessive "liver energy" accumulating at the patient's head. As kidneys are important for the brain nourishment, the deficiency of "kidney energy" would weaken the brain, which the latter naturally diminished with ages. When this was accompanied with "Liver Yang Rising" in brain, stroke might result (Gong and Sucher, 1999; Veith, 2002).

The diagnoses of TCM are through inspection and auscultation, inquiry, olfaction, and palpation (Liangyue *et al.*, 1987). The internal symptoms of wind stroke could be identified by pulse diagnosis. The TCM doctor could detect a "choppy" pulse, which is characterized by 3-5 dormant irregular slow pulses per respiration (Flaws, 1995; Maciocia and Ying, 1994).

Over a hundred of different Chinese medicines had long been used for stroke prevention and treatment according to the body nature of patients, aiming to restore the balance of external and internal winds (Zhou and Xiao, 1997).Numerous Chinese herbal medicine or herbal formulae had been proven to have anti-inflammatory, anti-thrombotic and neuroprotective effects (Grilli *et al.*, 1996; Huang *et al.*, 1994a). Besides, the thousand-year long experience of TCM have so far shown the effectiveness and with few or no side-effects in stroke therapy (Xu, 1991).

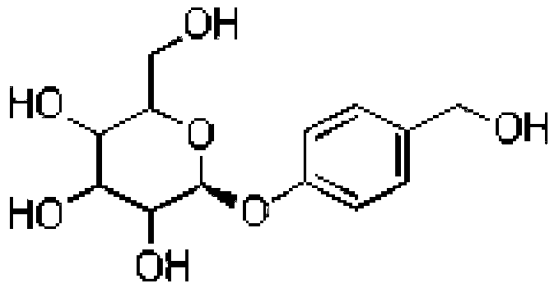
1.2.2 Tianma

Tianma, known as Gastrodiae Rhizoma, the dried root of *Gastrodia elata* blume (GEB), belonging to *Orchidaceae* family was commonly used in TCM. According to the Pharmacopoeia of the People's Republic of China (PPRC), the nature and flavor for Tianma are neutral ("*ping*") and sweet ("*gan*"), respectively (National Pharmacopoeia Committee, 2010). In TCM clinical practice, Tianma was usually used to cure neurasthenia, neurasthenic syndrome, headache, rheumatism, paralysis, hemiplegia and epilepsy (Wu *et al.*, 2007). It could be used in formulae with other herbs, such as "Qiang Li Tian Ma Du Zhong Wan" for expulsion of wind during stroke rehabilitation, "Tianma Gouteng Yin Formula" for anti-hypertension, etc.

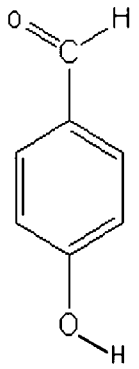
Besides, modern pharmacological studies suggested that Tianma had the effects of analgesia, improvement of microcirculation, anti-hypertension, enhancing

blood vessel functions, anti-inflammatory and immunostimulation (Bulpitt *et al.*, 2007; Bulpitt, 2005; Feng *et al.*, 2012; Kim *et al.*, 2003).

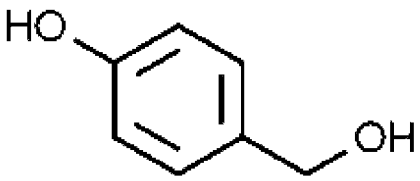
Major chemical components of Tianma had been identified including gastrodin, 4-hydroxybenzyl alcohol, vanillyl alcohol, vanillin, 4-hydroxybenzylaldehyde, glucose β-sitosterol, etc. (Baek *et al.*, 1999; Wu *et al.*, 2007; Wu *et al.*, 1989). The main active ingredients are gastrodin (GAS) (Figure 1.1), 4-hydroxybenzylaldehyde (4-HBAL) (Figure 1.2), 4-hydroxybenzyl alcohol (4-HBA) (Figure 1.3), vanillyl alcohol (Figure 1.4) and vanillin (Figure 1.5) (Zhou, 2011). The chemical structures of above chemical constituents were shown in Figure 1. GAS was known as the standard marker as stated in the PPRC (National Pharmacopoeia Committee, 2010).



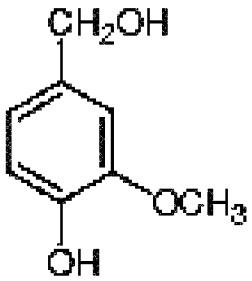
(a) Gastrodin



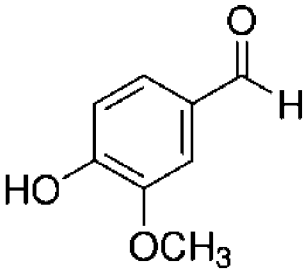
(b) 4-hydroxybenzylaldehyde



(c) 4-hydroxybenzyl alcohol



(d) Vanillyl alcohol



(e) Vanillin

Figure 1.1 Chemical structures of major chemical components of Tianma (a) Gastrodin, (b) 4-hydroxybenzylaldehyde, (c) 4-hydroxybenzyl alcohol, (d) Vanillyl alcohol and (e) Vanillin.

Both Tianma organic crude extracts and isolated ingredients such as GAS and 4-HBA showed positive neuroprotective effects towards ischemia (Kam *et al.*, 2011; Kim *et al.*, 2007; Tsai *et al.*, 2011; Yu *et al.*, 2010; Zeng *et al.*, 2006). Besides, GAS was found to inhibit the vascular smooth muscle cell (VSMC) proliferation, which played a vital role in preventing arteriosclerosis and restenosis after treatments such as coronary intervention or vein grafting (Zhu *et al.*, 2012). The Banxia Baishu Tianma Tang (Decoction of *Pinellia ternata*, *Atractylodes macrocephala* and *Gastrodia elata*) was found to have an anti-hypertension effect (Wang and Xiong, 2011). In recent years, many studies have been done on Tianma, showing that it could improve the cognitive functions, memory and learning performance in mice (Chen *et al.*, 2011; Hsieh *et al.*, 1997; Shin *et al.*, 2011; Wu *et al.*, 2007). There were further investigations on whether Tianma had the therapeutic effects on neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, etc. (Choi, 2013; Mishra *et al.*, 2011). GAS was one of the natural calcium antagonists (Shu *et al.*, 2012) that could act as a calcium channel blocker and prevented intracellular Ca^{2+} ions from overloading (Xu *et al.*, 2007; Zeng *et al.*, 2006).

Oxidative stress and occurrence of free radicals during reperfusion would lead to massive cell deaths. Therefore, anti-oxidative and free radical scavengers were important properties for stroke therapy. The ethanolic extract and diethyl ether fractions of Tianma have been proven to function as an antioxidant and free radical scavenger for hydroxyl radical (OH ·), respectively (Kim *et al.*, 2007; Liu and Mori, 1992). For the active ingredients, Vanillyl alcohol showed an antioxidant effect during epileptic seizure in rats (Hsieh *et al.*, 1999). 4-HBA could inhibit oxidative stress as well as excitotoxicity, thus reducing neuronal death in CA1 region related to the upregulation of antioxidant related gene expressions (PDI and 1-Cys Prx) by 4-HBA (Yu *et al.*, 2005).

The excitotoxicity of stroke was closely related to the GABA, glutamate and NMDA concentration. The ether fraction of methanolic extracts of Tianma could readjust the decreased GABA and elevated glutamate concentration (Ha *et al.*, 2000). This finding was coherent to the result of GAS. It was reported that GAS could increase the GABA concentration probably by inhibiting the GABA shunt (An *et al.*, 2003).

Anti-inflammatory effects were also found in Tianma ethanolic extract and its active ingredients. The *in vitro* experiments showed that the Tianma ethanolic extract could inhibit NO production and downregulated the iNOS and COX-2 expression in a dose-dependent manner (Ahn *et al.*, 2007). This might be explained by the results from GAS and 4-HBA. GAS has been proven to inhibit the expression of iNOS, COX-2 and proinflammatory cytokines via MAPK pathways, as well as downregulating downstream NF-κB and cyclic AMP-responsive element-binding protein (CREB) by blocking the phosphorylation due to LPS-stimulation (Dai *et al.*, 2011). Similar results of inhibition of the expression of iNOS, COX-2 were also observed in 4-HBA (Lim *et al.*, 2007).

The anti-apoptotic effect was also observed in Tianma ethanolic extract. One possibility was the protective mechanism mediated by the A2A-R/ cAMP/ PKA/ CREB- dependent pathway. The serum deprivation-induced apoptosis was inhibited by increment of cAMP formation, PKA activity and CREB phosphorylation due to Tianma ethanolic extract (Tsai *et al.*, 2011). Another possible explanation was that Vanillin and 4-HBAL could inhibit both intracellular rise of Ca^{2+} and apoptosis induced by glutamate (Lee *et al.*, 1999b). The roles played by Bcl-2 and caspase-3 in neuronal death during experimental stroke were well known (Adams and Cory, 1998; Kumar, 2006). In that study, the antiapoptotic Bcl-2 expression was enhanced while the caspase-3 was downregulated by 4-HBA compared to the control group after

Middle Cerebral Artery occlusion (MCAo) (Yu *et al.*, 2010).

1.3 Aim of study

Tianma is a traditional Chinese herbal medicine for treating neuronal diseases such as paralysis, epilepsy and stroke. Besides, the active ingredients of Tianma, such as GAS, 4-HBA had been proven to have neuroprotective effects on various ischemic stroke cell injury pathways. We hypothesized here that Tianma water extract could be neuroprotective during ischemic stroke. No researches have been performed on the neuroprotective effects of Tianma water extract and therefore this study aimed at evaluating the neuroprotective effects of Tianma water extract towards cerebral ischemia. The following studies would be carried out:

1. To authenticate Tianma by TLC and quantify of chemical markers such as GAS and 4-HBAL in Tianma water extract by LC-MS.
2. To evaluate the neuroprotective effect of Tianma water extract on neuronal cells after ischemia-reperfusion by using an *in vivo* MCAo rat model to mimic the condition of cerebral stroke and to provide evidence for *in vitro* mechanistic studies.
3. To elucidate the neuroprotective mechanisms of Tianma by studying its anti-oxidative, anti-inflammatory and anti-apoptotic effects.

Chapter 2 Preparation of Tianma water extract

2.1 Introduction

Tianma (Gastrodiae Rhizoma; Chinese Name:天麻), the dried rhizome of Gastrodia elata blume of the Orchidaceae family and purchased from Zhixin Ltd., was grown in Hubei province, China. The voucher specimen of Tianma was kept at the museum of Institute of Chinese Medicine, The Chinese University of Hong Kong, with voucher specimen number 2011-3351. (Figure 2.1a).

(a) (b)

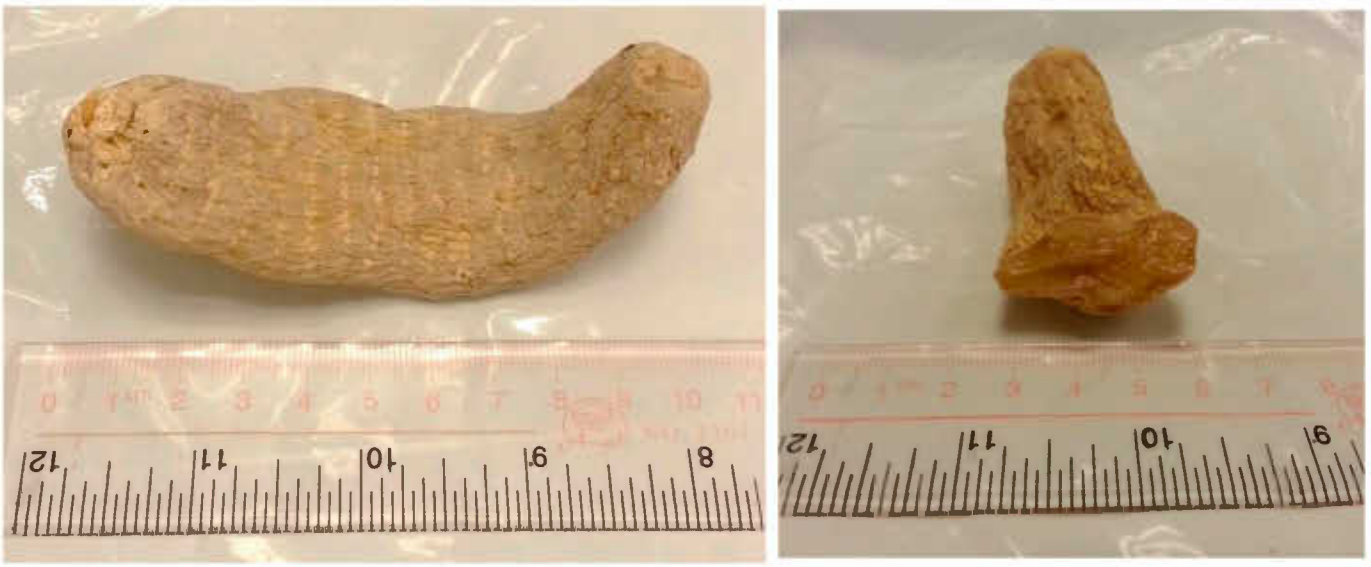


Figure 2.1 Raw herb of Tianma used in the study (a) An intact piece of Tianma (b) The cross section was shown.

2.2 Morphological and Chemical Authentication of Tianma (TLC)

The parameters for morphological authentication were adopted according to the Pharmacopoeia of the People's Republic of China 2010 (PPRC) (National Pharmacopoeia Committee, 2010). Tianma is a short (length: 3-15 cm; width: 1.5-6 cm), flat (thickness: 0.5-2 cm) cylinder root of slight curves with pale yellowish-white to light yellow rough surface. Wrinkles in both longitudinal and horizontal (surrounding the root) directions can be found. Tianma was cut horizontally and the texture was hard. The cut surface was smooth and appeared yellowish-white to light brown (Figure 2.1b).

Thin layer chromatography (TLC) was carried out for the chemical authentication for Tianma by comparing with the reference herb and standard marker (Gastrodin, GAS) purchased from National Institute for the Control of Pharmaceutical and Biological Products (Product no: 0944-200006, Beijing, China) according to the PPRC 2010.

The test solutions were prepared as follow. 0.5 g of raw herb and Tianma reference herb were weighed. 5 mL methanol was added to each sample and sonicated for 30 mins. 1 mg of standard marker GAS was dissolved in 1 ml methanol. 5 μL each of raw herb, Tianma reference herb and GAS test solution were loaded on TLC plate (Silica gel 60 F_{254}, Merck, Germany) in separated spots. The plate was then placed into a TLC tank pre-saturated with developing solvent (Ethyl acetate: Methanol: Water = 9:1:0.2). After the TLC chromatogram had developed up to 8 cm from the initial spot, the TLC plate was removed from the tank. The plate was observed under visible light after spraying evenly with indicator (5% phosphomolybdic acid in 50% ethanol). GAS was present among three samples with same $R_f = 0.24$ (Figure 2.2). This indicated that the raw herb purchased for our study was authentic Tianma.

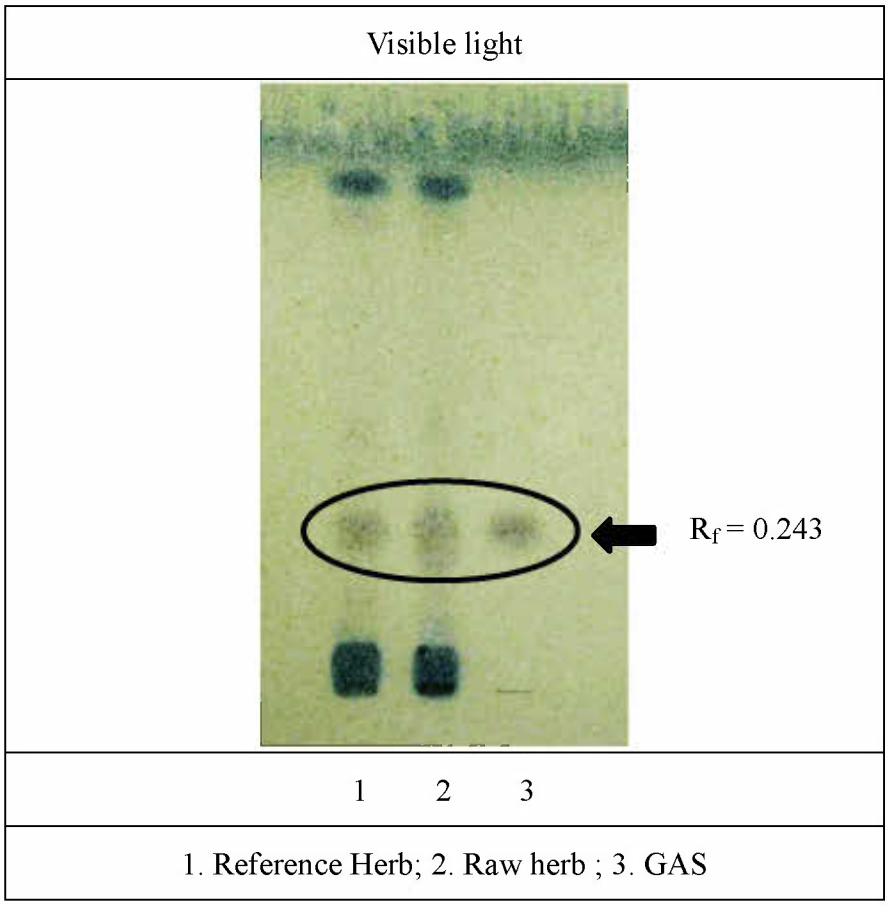


Figure 2.2 Details of the TLC profile of Tianma raw herb. TLC chromatogram of raw herb against standard marker, GAS and reference herb by using developing system Ethyl acetate: Methanol: Water = 9:1:0.2. An indicator (5% phosphomolybdic acid in 50% ethanol) was sprayed evenly on the TLC plate (Silica gel 60 F_{254}) to visualize the bands. The TLC was observed under visible light after applying indicator. The circle indicated the presence of GAS among three tested samples.

2.3 Preparation of Tianma water extract

200 g raw herb of Gastrodiae Rhizoma was allowed to soak with 2 L distilled water in the ratio of 1:10 (w/v) for 0.5 hour. Water extraction was carried out at 100 ^{o}C for 30 mins. The first round extract was collected. The herb was further extracted with 2 L distilled water in the ratio of 1:10 (w/v) for another 30 mins at 100 ^{o}C. After extraction, the water extract from both rounds were combined and filtered with cotton. The filtrate was concentrated under reduced pressure of -0.07 MPa at 60 °C. The concentrated extract was then lyophilized to dryness to give the dry powder (Figure 2.3). The weight of dried Tianma water extract collected was recorded for the estimation of the extraction yield. The extraction yield was 27.33 % w/w. The powder was stored at 4 ^{o}C for further use. Tianma water extract will be re-dissolved in distilled water or cell culture medium prior to the experiments. The same batch of powder extract (batch number: GR10042012-H2O) was used in both *in vitro* and *in vivo* experiments.

Figure 2.3 Dried powder of Tianma water extract

2.4 TLC and Chemical analysis (LC-MS) of Tianma extract

As gastrodin is known as the major active ingredient in Tianma, we also performed another TLC to investigate whether the GAS was present in our Tianma extract after extraction by comparing with the reference herb and standard marker (Gastrodin) purchased from National Institute for the Control of Pharmaceutical and Biological Products (Product no: 0944-200006, Beijing, China). The parameters of the TLC were same as described in Chapter 2.2 except the developing solvent (Ethyl acetate: Methanol: Water = 7:3:1). The details of the TLC chromatogram of Tianma extract were shown in Figure 2.4. GAS was present at the corresponding position on the TLC plate with the standard marker (R_f = 0.409). This indicated that presence of GAS in the Tianma extract we prepared.

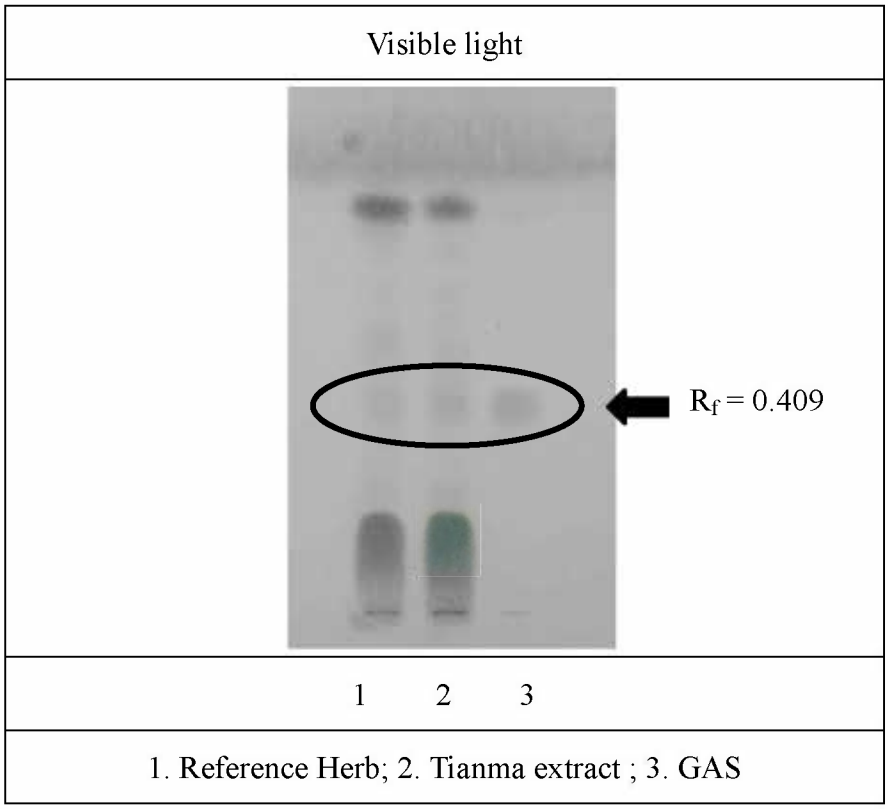


Figure 2.4 Details of the TLC profile of Tianma water extract. TLC chromatogram of Tianma extract against standard marker, GAS and reference herb by using developing system Ethyl acetate: Methanol: Water = 7:3:1. An indicator (5% phosphomolybdic acid in 50% ethanol) was sprayed evenly on the TLC plate (Silica gel 60 F_{254}) to visualize the bands. The TLC was observed under visible light after applying indicator. The circle indicated the presence of GAS among three tested samples.

In order to investigate the composition of the Tianma water extract, the chemical profile of Tianma water extract was examined against chemical markers such as GAS and 4-hydroxybenzaldehyde (4-HBAL) using liquid chromatography-mass spectrometry (LC-MS) (6530 accurate-mass Q-TOF LC/MS, Agilent Technologies, USA). The preparation of chemical markers was as followed. Both GAS and 4-HBAL were dissolved in MiliQ-H_2O (Millipore, Bedford, MA, USA) at 200 μg/mL and 1 μg/mL, respectively. A series of working solutions of GAS (3.125, 6.25, 12.5, 25, 50, 100, 200 μg/mL) were obtained by further diluting the stock solution, while another one for 4-HBAL were 0.0625, 0.125, 0.25, 0.5, 1μg/mL. A standard curve was used by establishing the average of six data point per concentration to investigate the concentration for GAS and 4-HBAL. All calibration curves had the correlation value $\geq$ 0.997. The 2 mg/mL Tianma water extract was prepared by dissolving in MiliQ-H_2O as well. Samples were filtrated by 0.2 μm filter (Millipore, USA) and 5 μl was injected into a ZORBAX Eclipse Plus C18 column (3x100 mm id, particle size 3.5 μm) (Agilent Technologies, USA) in separate analysis.

A gradient elution was carried out using the following solvent systems: mobile phase A- double distilled water/formic acid (99.9/0.1; v/v) and mobile phase B-acetonitrile. The linear gradient elution system was 97.5 % A to 30 % A for 15 minutes, then eluted with 5% A for 3 mins, finally equilibrated to 97.5 %A for 2 mins before next injection. The flow rate was set at 0.2 mL/min. The chemical markers identification was carried out by comparing the retention times of unknown peaks with those of the standard markers with matched ionization products' size.

Data was acquired in the negative-ion mode with Electrospray ionization (ESI) interface. The theoretical *m/z* values of the parent and product ions $[M-H]^-$ were set at *m/z* 285.1 to 123.0 for GAS, and $[M-H]^-$ at *m/z* 121.0 to 120.0 for 4-HBAL. The

Dual ESI parameters were set as follows: Dual ESI gas temperature, 350 °C; drying gas flow, 9 L/min; capillary voltage, 3500 V; the collision energy for GAS and 4-HBAL is ranged from 10 to 40 eV; acquisition rate and time use 6 spectra/s and 166.7 ms/spectrum, respectively. The transients/spectrum was 1640. The LC-MS data were analysed by the MassHunter Workstation Software Quantitative Analysis (Version B.05.00 / Build 5.0.519.0 Agilent Technologies, USA). After analysing the selected chemical markers GAS and 4-HBAL, we found their retention time were 4.642 min and 8.741 min respectively. According to this retention time, we further quantitated the compounds with the same retention time in Tianma water extract. The LC-MS profile and quantitative analysis of GAS and 4-HBAL in Tianma water extract were shown in Figure 2.5 and 2.6 respectively.

The quantitated percentages of GAS and 4-HBAL in Tianma water extract were 2.87% (w/w) and 0.00309% (w/w), respectively (Table 2.1), which was comparable to some previous studies stated that the GAS and 4-HBAL in water extract from grounded Tianma are 0.15% and 0.003% respectively in HPLC analysis (Liu *et al.*, 2002b).

In conclusion, the TLC showed the Tianma raw herb purchased was authentic. The presence of GAS in the Tianma water extract was confirmed by TLC chromatography. Besides, the LC-MS profiles of GAS and 4-HBAL revealed their quantities in Tianma water extract.

Table 2.1 Content of active ingredients in the Tianma extract by LC-MS analysis

Compound in extract	Concentration in 2 mg/mL Tianma extract (mg/mL)	% of compound in Tianma extract (w/w)
Gastrodin (GAS)	0.05733 mg/mL	2.87%
4-hydroxybenzaldehyde (4-HBAL)	0.06180 μg/mL	0.00309%

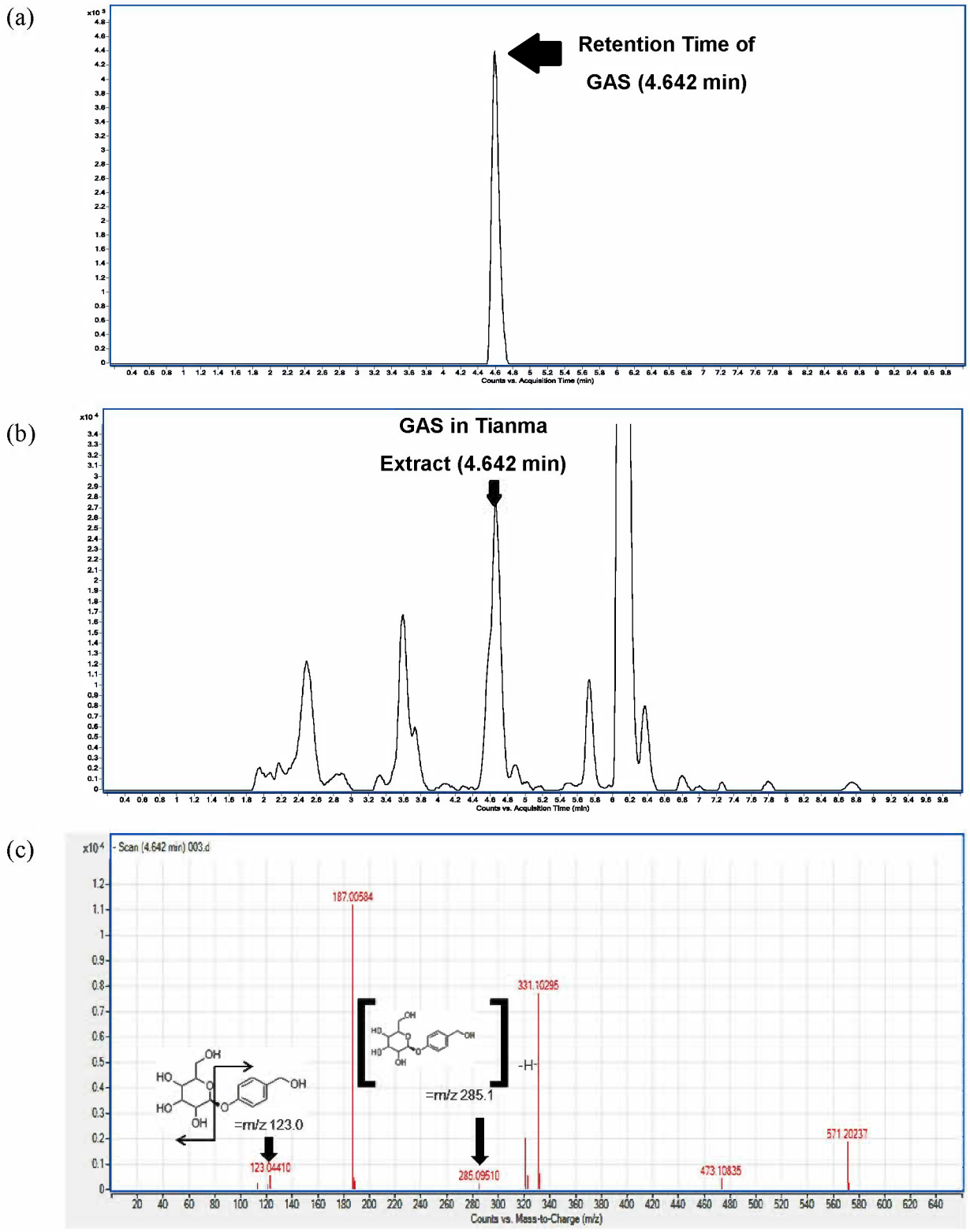


Figure 2.5 Representative LC-MS profile of (a) standard GAS, (b) Tianma water extract used in the study from retention time 0 s to 15 s and (c) Mass spectrum of GAS fragmentation (parent ion and daughter ions) identified in Tianma water extract.

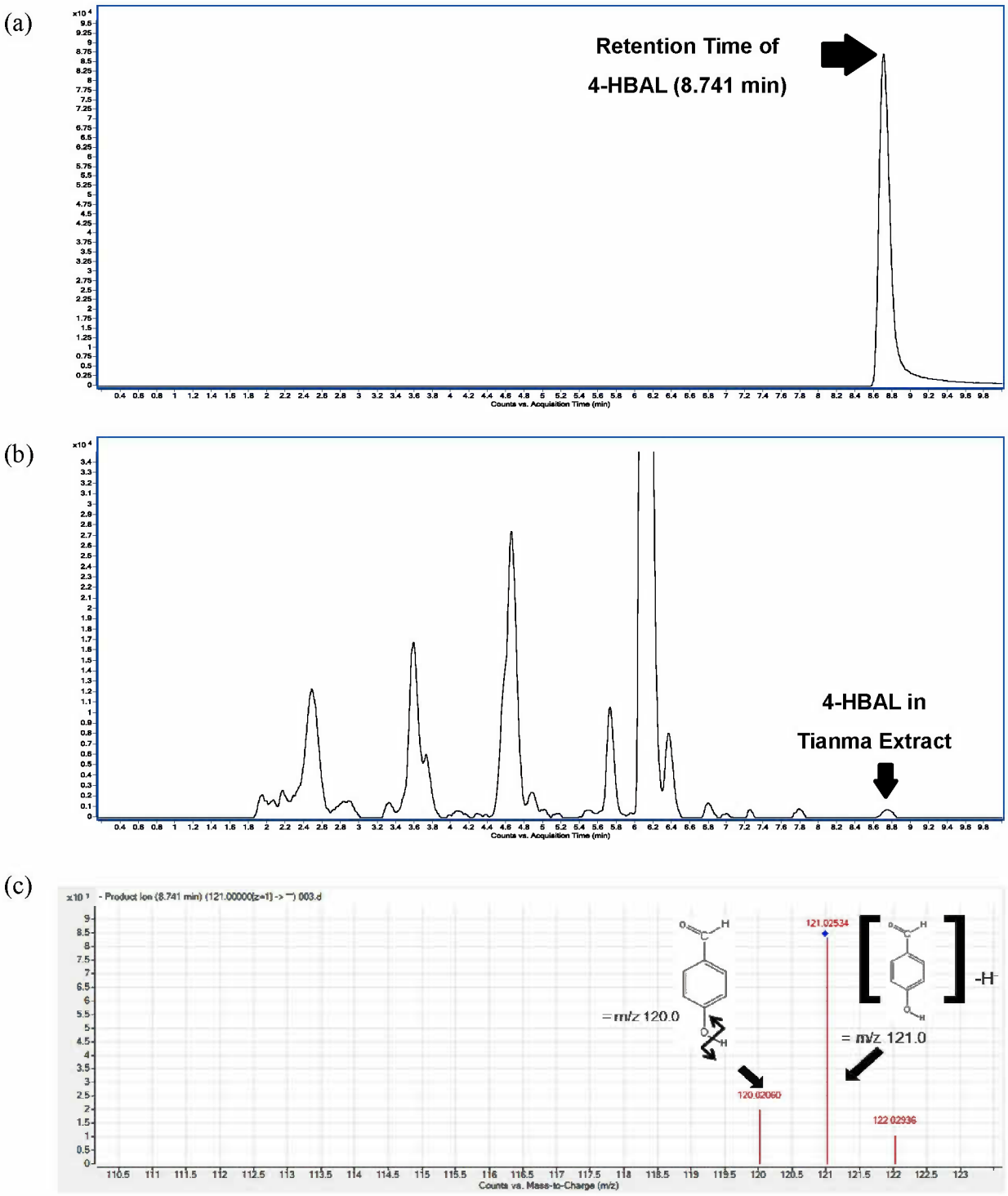


Figure 2.6 Representative LC-MS profile of (a) standard 4-HBAL, (b) Tianma water extract used in the study from retention time 0 s to 15 s and (c) Mass spectrum of 4-HBAL fragmentation (parent ion and daughter ions) identified in Tianma water extract.

Chapter 3 Protective effect of Tianma extract on *in vivo* cerebral ischemia (MCAo)

3.1 Introduction

All the animal experimental procedures in this study were approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong and the Department of Health of the government of the Hong Kong Special Administrative Region.

Ischemic stroke is referred to as a sudden loss of brain function due to a blockage of blood vessels in the brain, resulting in neuronal cell deaths (American Heritage Medical Dictionary, 2000). Stroke can be characterized by various symptoms, such as aphasia, hemiparesis, etc. (Brott *et al.*, 1989; Gelmers *et al.*, 1988).

As mentioned before, about 80% ischemic strokes in human occurred in the middle cerebral artery (MCA) or its branches (De Freitas *et al.*, 2008; Durukan and Tatlisumak, 2007). Intra-arterial suture occlusion of the middle cerebral artery (MCAo) model established by Kozuimi *et al.* (Koizumi *et al.*, 1986) in 1980s has been a widely used cerebral ischemia rodent model for neuroprotective studies (Cui *et al.*, 2010; Kang *et al.*, 2006; Wang *et al.*, 2003). The middle cerebral artery occlusion in this model was achieved by insertion of an intraluminal filament (suture) at the origin of the MCA without craniectomy (Longa *et al.*, 1989; Schmid-Elsaesser *et al.*, 1998). The evaluations of neurological impairment after ischemic stroke could be assessed by brain infarction volume and neurological score.

There are numerous cell injury pathways triggered when ischemia occurred and during reperfusion. Oxidative stress due to the formation of free radicals would lead to the generation of superoxide and nitric oxide (Huang *et al.*, 1994b; Siesjo *et al.*, 1995). This would lead to neuronal cell death and brain infarct during ischemia and

reperfusion (Chan, 1994; Özben, 1998; Siesjö *et al.*, 1989). The free radicals formed were mainly scavenged by anti-oxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Chan, 1996).

Inflammation was also involved in ischemic stroke neuronal cell injury (Doyle *et al.*, 2008) due to the presence of inflammatory mediators such as cytokines (Wood and Rothwell, 1998). After ischemia, pro-inflammatory cytokines, Interleukin (IL)-1β and tumor necrosis factor (TNF)-α were upregulated (Wood and Rothwell, 1998) and exacerbated ischemic injury, such as causing cerebral edema (Gordon *et al.*, 1990) and increased the permeability of blood-brain barrier or even exerted direct toxicities to capillaries (Beutler and Cerami, 1987; Goldblum and Sun, 1990).

The western conventional treatment of ischemia was focusing on perfusion and reduction of neuronal cell death (Ferriero, 2004; Moskowitz *et al.*, 2010). However, patients might have a higher risk of having intracerebral hemorrhage after recombinant tissue-type plasminogen activator (rt-Pa) treatment (NINDS rt-PA Stroke Study Group, 1995). No drug for stroke treatment targeting on anti-oxidation, anti-inflammation and anti-apoptosis pathways have acquired USFDA approval for acute ischemic stroke treatment yet (Roach *et al.*, 2010). Hundreds of Chinese medicines had long been used for stroke prevention and treatment (Zhou and Xiao, 1997) and had been proven to have neuroprotective effects (Huang *et al.*, 1994a), yet with only few or no side-effects (Xu, 1991). Moreover, some active ingredients in Tianma had been proven to be neuroprotective after MCAo (Hong, 2009; Yu *et al.*, 2010), but no related studies on Tianma water extract have been performed.

The neuroprotective effect of Tianma water extract on the ischemia-reperfuion in MCAo rat model will be reported in this Chapter. Rats were pretreated with Tianma water extract 1 hour before performing the MCAo and the ischemia lasted for 2 hours, followed by 24 hours of reperfusion. The brain infarction and

neurological score would be investigated as well as the determination of anti-oxidative effect and anti-inflammatory effect of Tianma water extract by the study on anti-oxidative enzymes SOD, GPx and catalase as well as the pro-inflammatory cytokines TNF-α and IL-6.

3.2 Materials and methods

3.2.1 Tianma treatment

Male Sprague-Dawley (SD) rats with body weight 250-270 g were supplied by the Laboratory Animal Services Center (LASEC) of The Chinese University of Hong Kong. Rats were housed 3-4 per cage in a controlled environment with a 12-hour light-and-dark cycle. Food and water were supplied *ad libitum*.

The maximum clinical dose of Tianma stated in Pharmacopoeia of the People's Republic of China (PPRC) (National Pharmacopoeia Committee, 2010). is 10g for a 60 Kg human.

$$10g / 60\ Kg = 166.67\ mg / Kg$$

The dose was converted for rats according to the USFDA's guideline human equivalent dose (HED) by multiplying the clinical dose by 6.2 (FDA, 2005). The extraction yield of Tianma water extract was 27.33 % w/w. Therefore, the HED of Tianma was as follow,

$$166.67mg/Kg \times 6.2 \times 27.33\% = 0.282\ g/Kg$$

Rats were divided into sham and 3 MCAo-operated groups, including control, 0.3 g/Kg (round up from 0.282 g/Kg) Tianma extract (1X HED) and 1 g/Kg Tianma extract (3X HED) randomly. The control group was supplied with distilled water instead of Tianma extract as vehicle. Distilled water or Tianma extract was orally administered to the rats through a bulb tipped intragastric gavage needle attached to a syringe 1 hour before MCAo. Dry Tianma extract powder was freshly prepared by

dissolving in distilled water prior to use. For the sham operated group, rats were subjected to same MCAo operation procedure only but without the insertion of intraluminal filament into the blood vessel.

3.2.2 Induction of transient focal cerebral ischemia (TIA) by MCAo

3.2.2.1 Intraluminal filament

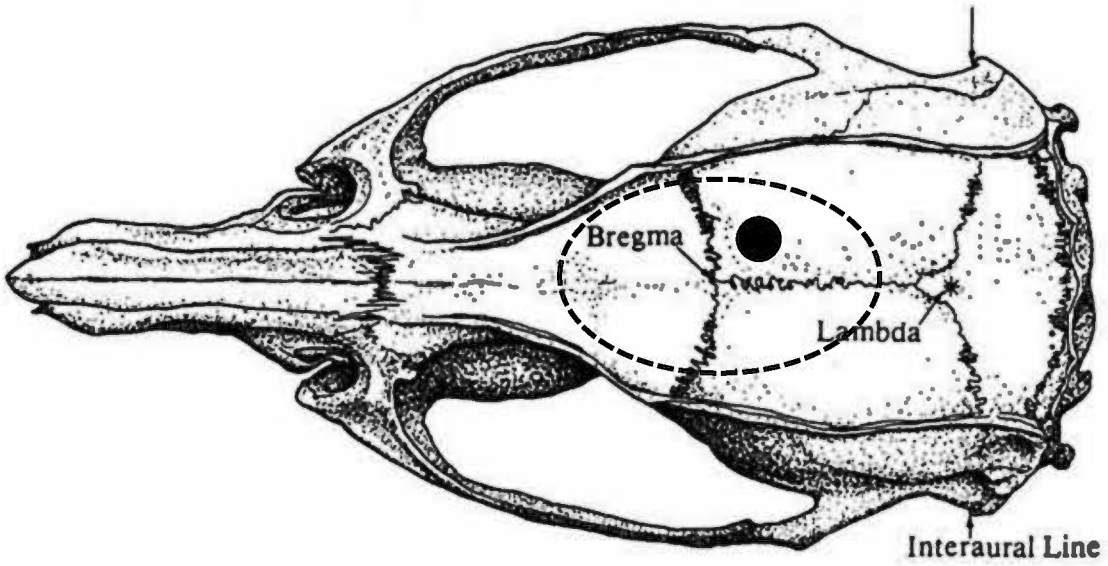
The AAAA grade poly-L-lysine coated intraluminal filament with a bulb head and a smooth cylindrical body for MCAo was used (#2838-100, Beijing Sunbio Biotech Co. Ltd., China). The head diameter and the cylinder were 0.38 ± 0.02mm and 0.28 mm respectively according to manufacturer. A mark on the filament was made at 18-20 mm which indicates the approximate length to be inserted into the external carotid artery (ECA).

Figure 3.1 The poly-L-lysine coated intraluminal filament head (0.38±0.02mm) with cylinder diameter of 0.28 mm as seen under microscope (40X magnification).

3.2.2.2 Cerebral blood flow measurement by laser Doppler flowmetry

The cerebral blood flow was monitored throughout the operation by a laser Doppler flowmeter (PeriFlux System 5000, Perimed AB, Stockholm, Sweden). Male Sprague-Dawley (SD) rats weighing 250-270 g were anesthetized by intraperitoneal injection of 400 mg/Kg chloral hydrate (VWR International Ltd., Poole, England) dissolved in phosphate-buffered saline (PBS). The skin over the skull was sterilized with 70% ethanol and a sagittal midline incision of 1 cm on the skin was made. The superficial fascia covering the skull was removed to expose the skull around the bregma. A probe holder (Probe holder PH07-6, Perimed AB, Sweden) for holding the laser Doppler probe (407; Perimed AB, Jarfalla, Sweden) was placed above the skull at the right cerebrum, 1 mm posterior and 2 mm lateral to Bregma, which was the area over the MCA (Gamboa *et al.*, 2010; Spratt *et al.*, 2006) (Figure 3.2a and b). The cerebral blood flow was monitored during the operation.

(a)



(b)

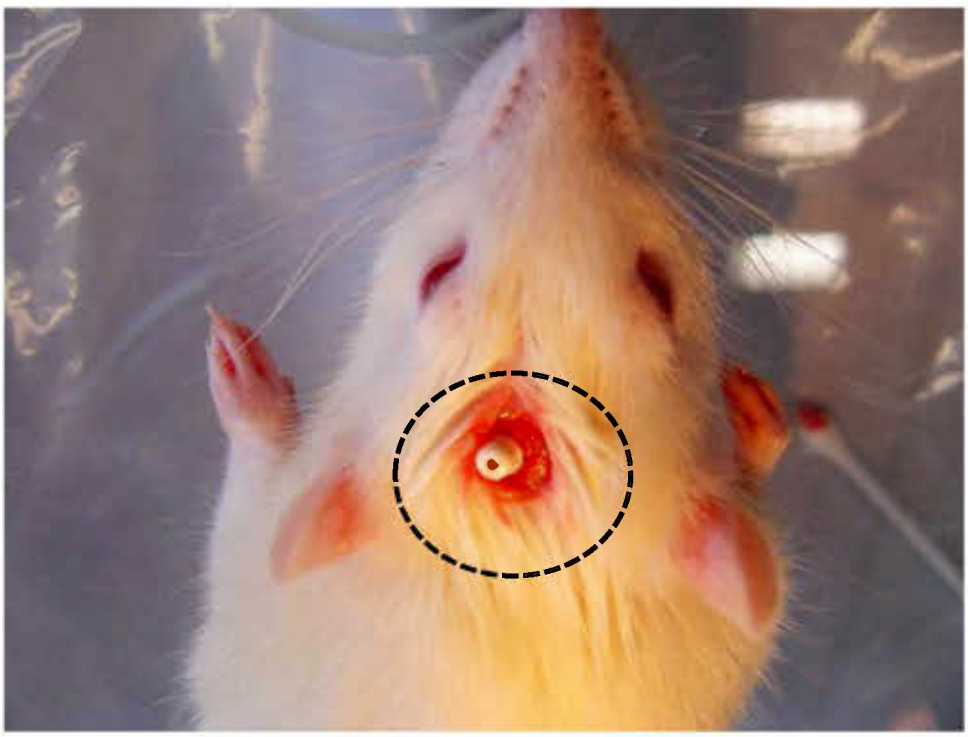


Figure 3.2 Location of the laser Doppler probe holder (a) as shown by the black spot, on the right hemisphere at 1 mm posterior and 2 mm lateral to bregma (Paxinos and Watson, 2006) (b) Location of the probe holder shown inside the black dotted circle

3.2.2.3 Middle cerebral artery occlusion (MCAo)

The MCAo was known to produce a transient focal cerebral ischemia, induced by the intraluminal filament without craniectomy, described by Longa *et al.* in 1989 (Longa *et al.*, 1989) (Figure 3.3) and modified by Kawamura *et al.* (Kawamura *et al.*, 1994). The MCA, common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) involved in this study were all on the right sides of the rats.

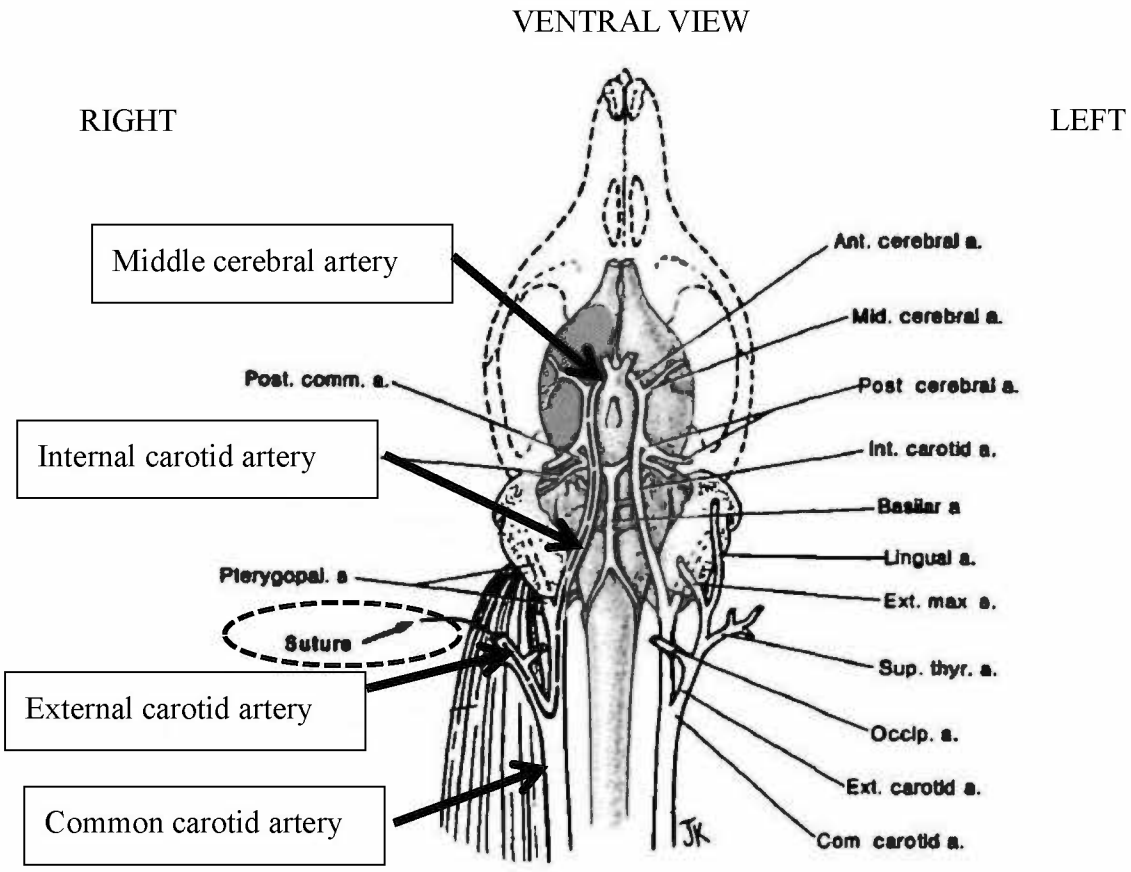


Figure 3.3 The procedure of MCAo in ventral view. The intraluminal filament (suture) was inserted from the right ECA and passed through the right ICA. The origin of the right MCA was occluded finally (Longa *et al.*, 1989).

The skin at the thoracic region of the rat was sterilized by 70% ethanol before making a ventral midline incision of about 1.5 cm. The trifurcation of right CCA, right ECA and right ICA were exposed. In order to stop accidental bleeding during the operation, a 4.0 silk braided suture (Kent Scientific Corporation, Torrington, Connecticut, USA) was placed beneath the CCA. Bleeding could be stopped by temporarily elevating this suture. There were a small bridge vessel between ECA and ICA, and also a small branch from the ECA. Both of these vessels were isolated and ligated by cautery (Gemini Cautery System, BIOSEB, Vitrolles, France). After that, the ECA was tightened up with a 4.0 silk braided suture and ligated by cautery at the far end of the ECA-ICA junction. The CCA and ICA were then clamped by microvascular clips (World Precision Instruments Inc., Sarasota, FL, USA) prior to using a pair of Vannas scissors (World Precision Instruments Inc., USA) to make a transverse incision at the ECA. The intraluminal filament was inserted into the ECA via this incision and pushed along ICA after crossing the trifurcation of the CCA, ICA and ECA. A 5.0 silk braided suture was tied loosely around the ECA for holding the intraluminal filament in position. After that, the microvascular clips on the ICA was removed to allow the further insertion of the filament for an extra 18-20 mm until the marking on the filament reached the ECA incision.

This was one of the markers for successful occlusion of MCA. The 5.0 silk braided suture on ECA was tightened to prevent bleeding. The microvascular clip on the CCA was removed after then. More than 60% cerebral blood flow (in arbitrary units) was blocked as observed in the laser Doppler flowmeter, indicated a successful blockage at the origin of the MCA. The intraluminal filament was held in position for 2 hours (ischemia) (Figure 3.4). The exposed thorax was covered by a piece a cotton soaked with 0.9% saline to prevent dehydration. Additional 70 mg/Kg Chloral hydrate was injected intraperitoneally to maintain anesthesia if necessary.

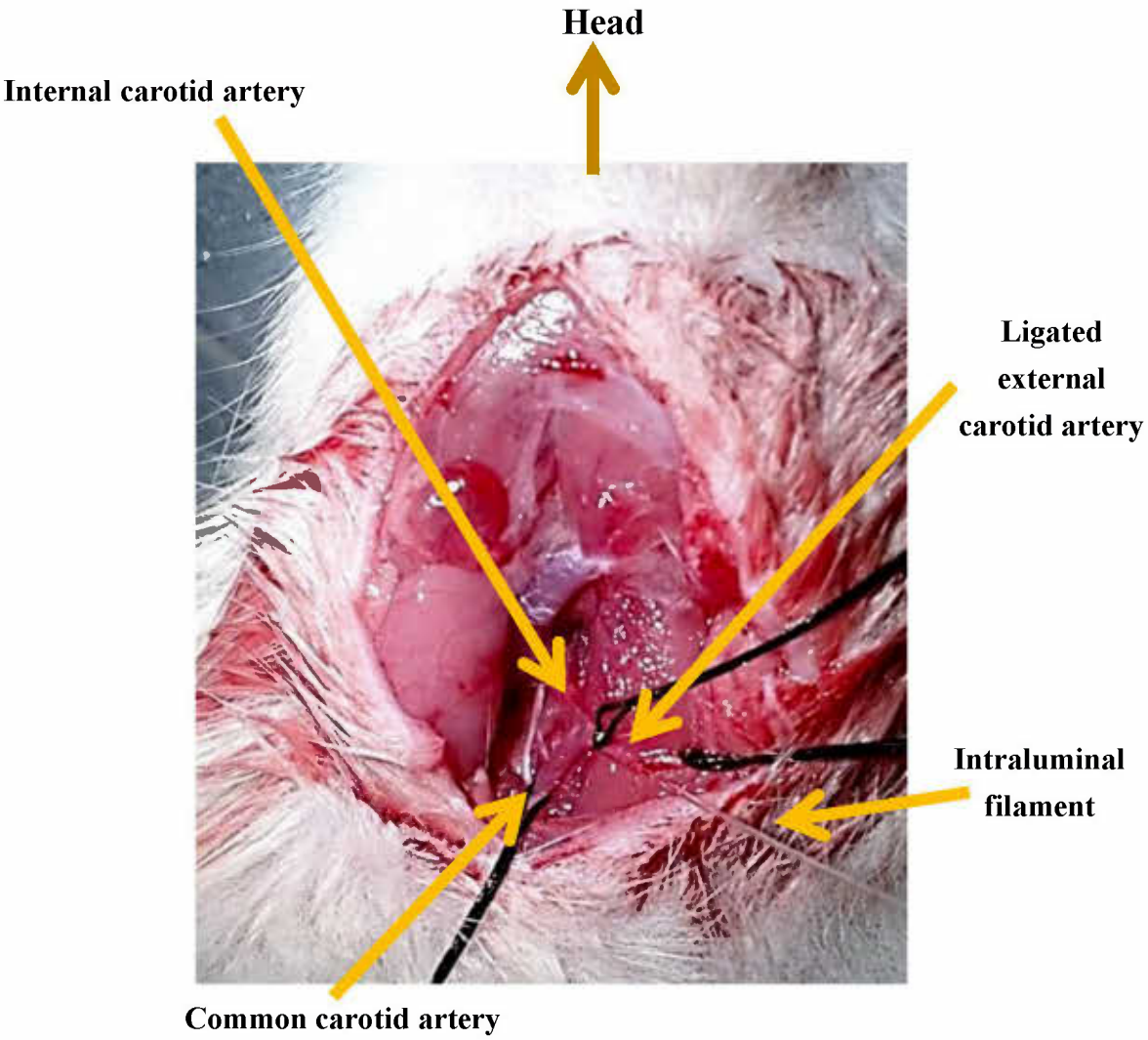


Figure 3.4 Insertion of suture during MCAo. The trifurcation of the CCA, ICA and ECA in rat at the thoracic region with inserted intraluminal filament.

The rats were subjected to ischemia for 2 hours and blood flow was restored

(reperfusion) by withdrawing the intraluminal filament from the ICA. After the intraluminal filament had been retracted, the ECA was tightened up with 5.0 suture. The wound at the thoracic region and the head was applied with Penicillin (10,000 units/mL) and streptomycin (10,000 μg/mL) (Invitrogen Corporation, Carlsbad, California, USA) and closed with 4.0 silk braided suture. After MCAo procedures, the rats were returned to the cages and housed individually. After 24 hours of reperfusion, rats were scored with a 4-point scale neurological scoring system (See section 3.2.3). The rats were sacrificed and plasma, brain, cerebrospinal fluid were collected for further analysis (See section 3.2.4-3.2.6).

3.2.3 Neurological scoring

As the motor cortex would be damaged after MCAo, the hemiparesis occurred (Nudo, 2006; Yonemori *et al.*, 1998). The neurological deficit of rats suffered from MCAo was measured by a 4-point scoring system modified from that described by Bederson *et al.* (Bederson *et al.*, 1986b) as shown in Table 3.1.. This scoring system was widely accepted by previous studies (Guan *et al.*, 2011; Kawai *et al.*, 2010; Kawamura *et al.*, 1991) for the investigation of behavioral change after MCAo. The MCAo was performed on the right cerebral hemisphere of the rat, so abnormal motor activities could be found on its contralateral side, i.e. the left side. Higher score implied more severe neurological deficits.

Table 3.1 A 4-point scale neurological scoring system for assessing the neurological deficit of rats

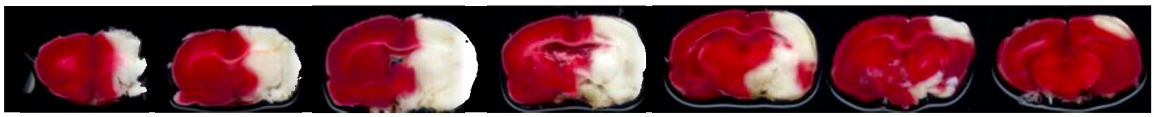
Neurological scores	Neurological deficit shown
0	No apparent neurological deficits
1	Contralateral forelimb flexion when pushed to the edge of a bench
2	Decreased resistance to lateral push to the left
3	Contralateral circling when pulled by the tail
4	Spontaneous circling or could not walk spontaneously

3.2.4 Brain infarction measurement by 2,3,5-Triphenyltetrazolium hydrochloride (TTC) staining

The rats were sacrificed with etherization. The brains were taken out and washed in cold PBS to remove blood. The brains were immersed in PBS and were frozen at -20 ^{o}C for 1.5 hours. A series of total 7 coronal slices in 2mm thickness, starting from 2 mm from the frontal pole, were obtained by using rat brain matrix (RBMA-300C, Kent Scientific Corporation, USA) (Figure 3.5).

Figure 3.5 The brain matrix for coronal brain slices preparation spaced 1 mm apart. The brain was placed on the matrix and sliced by blades by cutting at every 2-mm intervals. Photo adopted from manufacturer website.

2% 2,3,5-Triphenyltetrazolium hydrochloride (TTC) solution was prepared by dissolving the TTC powder (Sigma-Aldrich, St. Louis, MO, USA) in PBS. The slices were immersed in the solution and incubated for 15 minutes at 37 ^{o}C for 20 mins for staining. White indicated infarction while red staining indicated normal tissue (Figure 3.6) (Bederson *et al.*, 1986a; Lillie and Conn, 1969). The brain slices were fixed in 4% paraformaldehyde overnight.



Anterior Posterior

Figure 3.6 TTC-stained brain slices. White indicated infarction while red staining indicated normal tissue

The brain slices were captured by a desktop scanner (Epson Perfection 1260, Seiko Epson Corporation, Nagano, Japan) and a scanning software from the manufacturer (Epson TWAIN 5 version 5.60E, Seiko Epson Corporation, Japan). The fixed configuration of the program used for scanning was listed in Table 3.2.

Table 3.2 Configuration of the scanning software used for capturing images of the brain slices

Image type	**Resolution**	**Exposure**	**Gamma**
Color photo	600 dpi	6	1.32
Highlight	**Shadow**	**Saturation**	**Grey balance intensity**
162	46	15	25

The images were analyzed by image software (Adobe Photoshop CS5, Adobe Systems Inc., San Jose, CA, USA). Areas of brain slices were identified by the software. As the scanning resolution of 600 dpi (dots per inch) was configured and 1 inch was equal to 2.54 cm,

$$1\ cm^2 = \left(\frac{600\ dpi}{2.54}\right)^2 = 55800\ \text{pixels}$$

i.e. 55800 pixels represented 1 cm^2. This was applied to both areas stained in red and white. The individual infraction volume of brain slice was calculated as follow:

Individual infarction volume = Infarction area of each slice x thickness (2 mm)

Total infarction volume was calculated by summing up the individual infarction volume of all 7 slices.

3.2.5 Investigation on anti-oxidative enzymes activities and pro-inflammatory cytokines

3.2.5.1 Preparation of plasma, cerebrospinal fluid (CSF) and brain homogenates

Transient focal cerebral ischemia was induced with the same MCAo procedures as mentioned in section 3.2.2 for both control group and Tianma-treated group. Distilled water or Tianma extract was orally administered 1 hour before MCAo (See section 3.2.1). Same MCAo procedures were applied to the sham group, with surgery but without inserting the intraluminal filament. After 24 hours reperfusion, the rats were anesthetized by ether and blood was withdrawn. Plasma obtained by centrifuged (4000 x g, 15 mins) the heparinized blood at 4 ^{o}C. The cerebrospinal fluid (CSF) was collected by lumbar puncture. The skull was opened up and the brain was obtained and washed by ice cold PBS. The plasma and CSF were stored at -80 ^{o}C for further studies.

The brain homogenate was prepared with homogenized one gram of brain

tissue by a hand-held rotor-stator homogenizer (TissueRuptor, Qiagen, Germany) with 5mL tissue homogenizing buffer which contained 50 mM Tris-HCl (Sigma-Aldrich, USA), 5 mM EDTA (USB Cleveland, Ohio, USA) and 1 mM dithiothreitol (USB Cleveland, Ohio, USA). The homogenates were centrifuged at 10000 x g for 15 mins at 4°C. The supernatant (brain homogenate) was collected and aliquoted into small portions and was frozen at -80 °C for further analysis including measurement of anti-oxidative enzyme activities and pro-inflammatory cytokines.

3.2.5.2 Determination of anti-oxidative enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activities

Enzyme activity was normalized by protein amount. Protein concentration in the sample extracts was quantified by the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985). BCA working solution was prepared by mixing BCA solution (Sigma Aldrich, St. Louis, MO, USA) and Copper (II) Sulphate Pentahydrate ($CuSO_4$*5 H_2O) solution at the ration of 50:1. Bovine serum albumin (BSA) (Sigma-Aldrich, USA) was used as protein standard. Protein standards (0 µg/mL, 2µg/mL, 4µg/mL, 6µg/mL, 8µg/mL and 10µg/mL BSA) and samples each of 5 µL were added with 200 µL BCA working solution and incubated for 30 mins at 37 °C. The absorbance at 540 nm was measured by using a microplate reader (uQuant microplate spectrophotometer, BioTek Instruments, Winooski, VT, USA). The protein content of the samples was calculated with the BSA standard curve.

SOD activity was measured by the Superoxide Dismutase Assay Kit (Cayman Chemical Company, USA). The principle of the assay kit was shown in Figure 3.7.

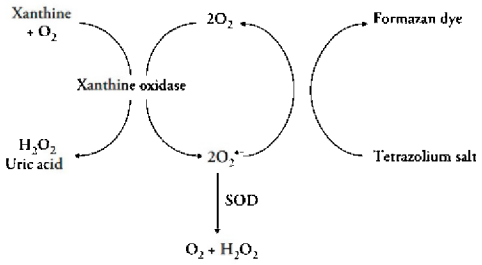


Figure 3.7 Principle of the SOD assay kit. Figure adopted from manufacturer kit booklet

Tetrazolium salt was utilized in this assay for detection of superoxide radicals generated by xanthine oxidase (XO) and hypoxanthine. The tetrazolium salt would be converted to formazan dye after reduction by a superoxide anion. The SOD present in brain homogenates should be able to remove the superoxide generated from XO and hypoxanthine. The remained superoxide not removed by the SOD would reduce the tetrazolium salt into the formazan dye. Thus SOD activity could be determined by measurement of the dismutation of superoxide radicals after incubation at 37°C for 20 minutes. The absorbance at 450 nm could be detected by using the microplate reader.

GPx activity was measured by the Glutathione Peroxidase Assay Kit (Cayman Chemical Company, USA). The principle of the assay kit was shown in Figure 3.8.

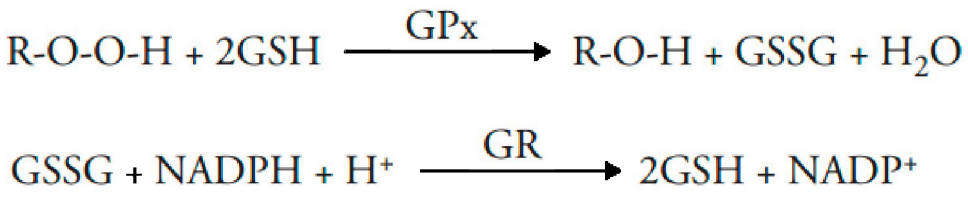


Figure 3.8 Principle of the GPx assay kit. Figure adopted from manufacturer kit booklet

The reduction of organic hydroperoxide (ROOH) with the oxidation of glutathione (GSH) was catalyzed by GPx and produced oxidized glutathione (GSSG) in return. GSSG is recycled to its reduced form, GSH by glutathione reductase (GR) and NADPH. This reduction was coupled with oxidation of NADPH to $NADP^+$, which was accompanied by a decrease in absorbance at 340 nm. The GPx activity in the sample was directly proportional to the rate of decrease in absorbance at 340 nm. In this assay kit, Cumene hydroperoxide was utilized as a substrate of the GPx. The kinectics (at absorbance 340 nm) was measured for 9 mins, at one minute time intervals and using a microplate reader.

CAT activity was measured by Catalase fluorometric detection kit (Enzo Life Sciences, Inc., USA). The principle of the assay kit was shown in Figure 3.9.

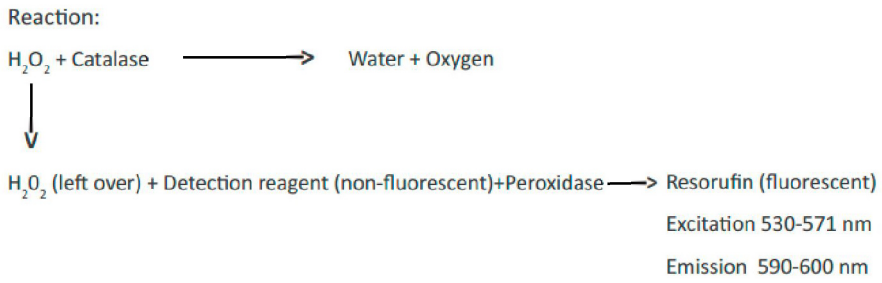


Figure 3.9 Principle of the CAT assay kit. Figure adopted from manufacturer kit booklet.

H_2O_2 was utilized as the substrate for CAT. A non-fluorescent detection reagent and horseradish peroxidase (HRP) would react with the left over H_2O_2 from the catalase reaction. After the brain homogenates were added to different wells of a black 96-well plate, 40 μM H_2O_2 solution was added and incubated for 30 mins at room temperature. The reaction cocktail (detection reagent and HRP) was added to the reaction mixture and further incubated for 15 mins at room temperature. The

H_2O_2 left behind in the sample wells would react with the HRP and detection reagent to yield fluorescent Resorufin. The concentration of Resorufin was detected by using a fluorescent microplate reader (FLUOstar OPTIMA, BMG LABTECH, Germany) with software (Fluoster Optima software Version 1.30-0, BMG LABTECH GmbH, Germany), measured at excitation of 535 nm and emission of 595 nm. The CAT activity was quantified by comparing the fluorescence against a CAT standard curve.

3.2.6 Measurement of pro-inflammatory cytokines, TNF-α and IL-6 in plasma, cerebrospinal fluid (CSF) and brain homogenates

BD OptEIA Enzyme-linked immunosorbent assays (ELISA) (BD Biosciecnces, San Diego, CA, USA) sets were used to measure the pro-inflammatory cytokines, TNF-α and IL-6.

The ELISA sets were used according to manufacturer's instruction. The protocol was summarized as follow. The capture antibodies of TNF-α and IL-6 were reconstituted in coating buffer as stated by the manufacturer. The plates coated with capture antibodies and were sealed. The wells were aspirated with wash buffer after overnight incubation at 4 ^{o}C. The wells were then soaked with assay diluent which was 10% Fetal Bovine Serum (FBS) (Gibco, Life Technologies, California, USA) in PBS for 1 hour at room temperature. The wells were aspirated with wash buffer again to remove the assay diluent.

Standard was diluted in assay diluent in 2-fold dilutions for a total of 6 concentration gradients for plotting the standard curve. The highest standard concentrations for TNF-α and IL-6 were 50000 pg/mL and 62500 pg/mL, respectively. 200 μL of each sample and standards were added to each well. The plates were sealed and incubated for 2 hours at room temperature. After the incubation, the wells were aspirated and washed with wash buffer. 100 μL of

detection antibodies, diluted in assay diluen, were added and incubated for 1 hour at room temperature. The wells were aspirated and washed with wash buffer again. 100 μL of enzyme reagent (Streptavidin-HRP), diluted in assay diluent, was added and incubated for 30 mins at room temperature. The wells were aspirated and washed with wash buffer thoroughly after incubation. TMB Substrate Reagent A and B (BD OptEIA, BD Biosciences, Becton, Dickinson and Company) were mixed in 1:1 v/v ratio to give TMB Substrate Solution. 100 μL of TMB Substrate Solution was added to each well and incubated for 30 mins further in the dark at room temperature. 50 μL of stop solution (2N H_2SO_4) was added to each well to stop the reaction. The absorbance at 450 nm was read by the microplate reader (uQuant microplate spectrophotometer) within 30 mins after the reaction was stopped.

According to the manufacturer, analytical sensitivities were 10 ng/mL and ≥ 10 ng/mL for TNF-α and IL-6 respectively. However, the actual performance of the TNF-α assay in this study had not reached the proposed level for our CSF samples. This might be due to the variation of set performance substantially from that cited by the manufacturer.

3.2.7 Statistical Analyses

The differences between the control and sham groups were tested with unpaired t-test unless specified. The differences between the control groups and different dose treatment groups were tested with one-way analysis of variance (one-way ANOVA) by Dunnett's multiple comparisons test except specified. All statistical analyses were performed at 5% level of significance ($p<0.05$) by using GraphPad Prism software version 6.01 (GraphPad Software, San Diego, California, USA). Data were expressed as mean ± standard error of the mean (SEM) in all *in vivo* experiments.

3.3 Results

3.3.1 Neurological score and Average infarct volume

The neurological behavioral defect was assessed by the 4-point scale proposed by Bederson (Bederson *et al.*, 1986b). The result was shown in Figure 3.10. The result of sham group was not shown in Figure 3.10 as all rats scored 0. The neurological score of the control, 0.3 g/Kg and 1 g/Kg Tianma-treated groups were 2.64 ± 0.15, 2.40 ± 0.18 and 1.85 ± 0.25, respectively. The neurological score was ameliorated in a dose-dependent manner in the Tianma-treated groups. The 1 g/Kg Tianma extract treated group showed a significant decrease ($p < 0.05$) in neurological score as compared with that of control.

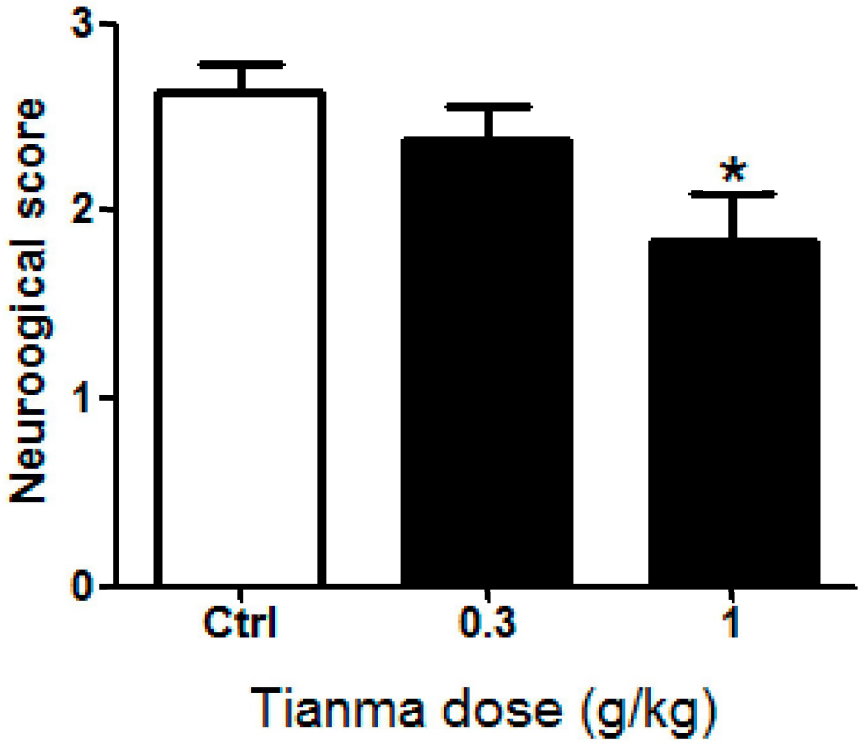


Figure 3.10 Neurological score of Tianma-treated rats after MCAo. Control group was fed with distilled water as vehicle intragastically. Neurological score of different groups of rats was evaluated 24 hour after reperfusion. Data were expressed as mean ± SEM. $n \geq 12$. *$p < 0.05$ as compared with control by one-way ANOVA.

Rats were sacrificed after neurological scoring. The brains were dissected out with care. After washing with PBS, brains were cut into coronal slices. TTC staining was carried out to evaluate average infarct volume (Figure 3.11a). The quantified brain infarct was shown in Figure 3.11b. All rats in sham group had no infarct and was not shown. The average brain infarct volume in the control, 0.3 g/Kg and 1 g/Kg Tianma-treated groups were 0.41 ± 0.04 cm^3, 0.27 ± 0.03 cm^3 and 0.24 ± 0.03 cm^3, respectively. The data was also expressed in percentage (Figure 3.11c). All rats in sham group had no infarct and was not shown. The average percentage of brain infarct in control, 0.3 g/Kg and 1 g/Kg Tianma-treated groups were 22.5 ± 8.28%, 15 ±6.55% and 12.5 ± 6.10% resepctively. A dose-dependent decrease in brain infarct was observed in the Tianma-treated groups. Both average brain infarct volume and percentage of brain infarct of 0.3 g/Kg ($p < 0.05$) and 1 g/Kg ($p < 0.01$) of Tianma-treated groups decreased significantly as compared with that of control.

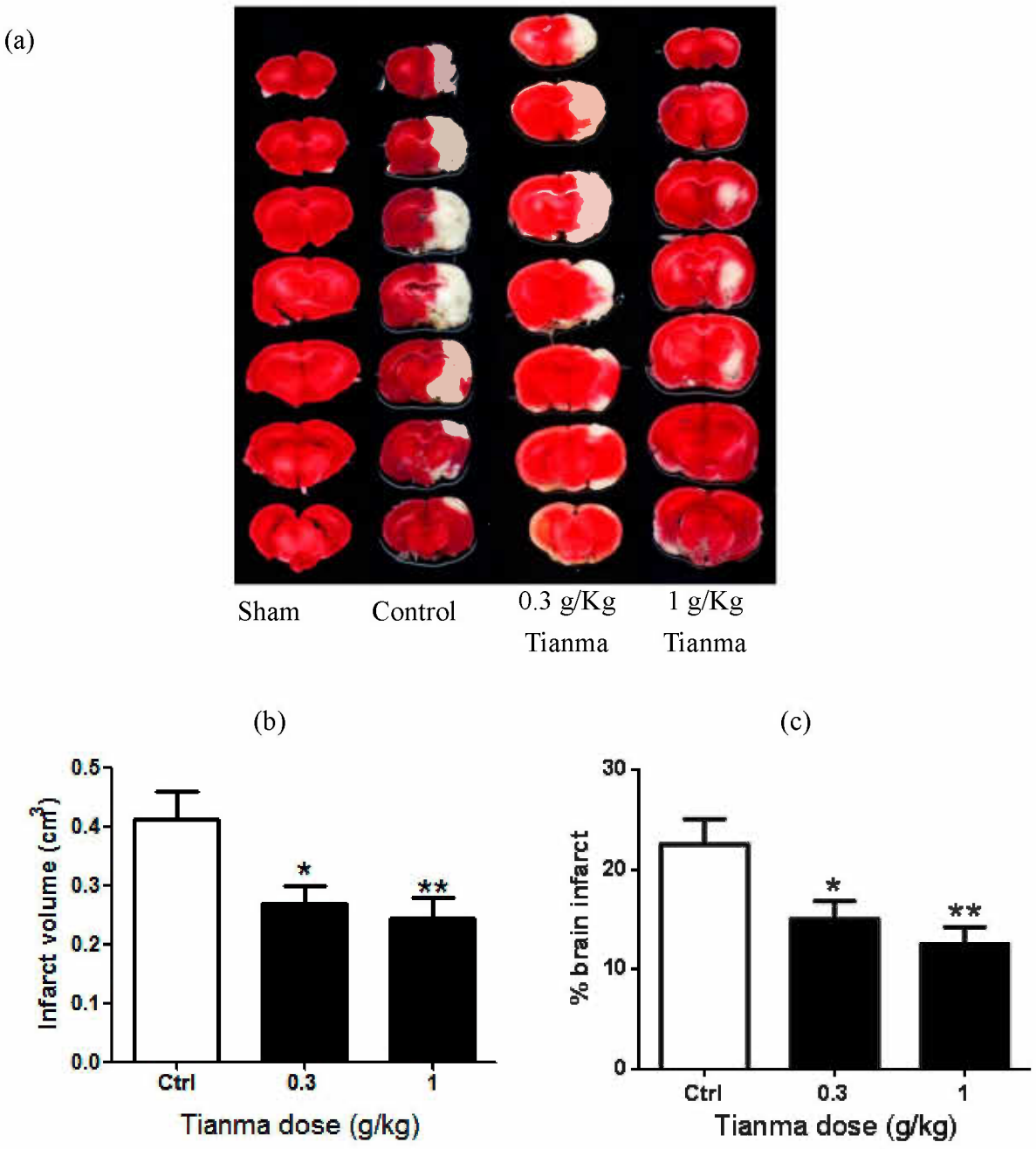


Figure 3.11 Brain infarct volume of Tianma-treated rats after MCAo. Control group was fed with distilled water as vehicle intragastrically. (a) Brain slices after TTC staining. (b) Average brain infarct volume and (c) average percentage of brain infarct of different groups of rats. Data were expressed as mean ± SEM. $n \geq 12$. *$p < 0.05$, **$p < 0.01$ as compared with control by one-way ANOVA.

3.3.2 Anti-oxidative enzyme activity

The SOD specific activity of the brain tissue of different groups was shown in Figure 3.12. The SOD specific activity of the sham, control, 0.3 g/Kg and 1 g/Kg Tianma-treated groups were 110 ± 4.6 U/mg protein, 37.4 ± 2.0 U/mg protein, 59.0 ± 2.6 U/mg protein and 75.1 ± 4.2 U/mg protein, respectively. The control group showed a significant decrease ($p < 0.001$) in the SOD specific activity as compared to the sham group. A significant increase in the SOD specific activity were observed in both 0.3 g/Kg ($p < 0.001$) and 1 g/Kg ($p < 0.001$) Tianma-treated groups as compared to the control group.

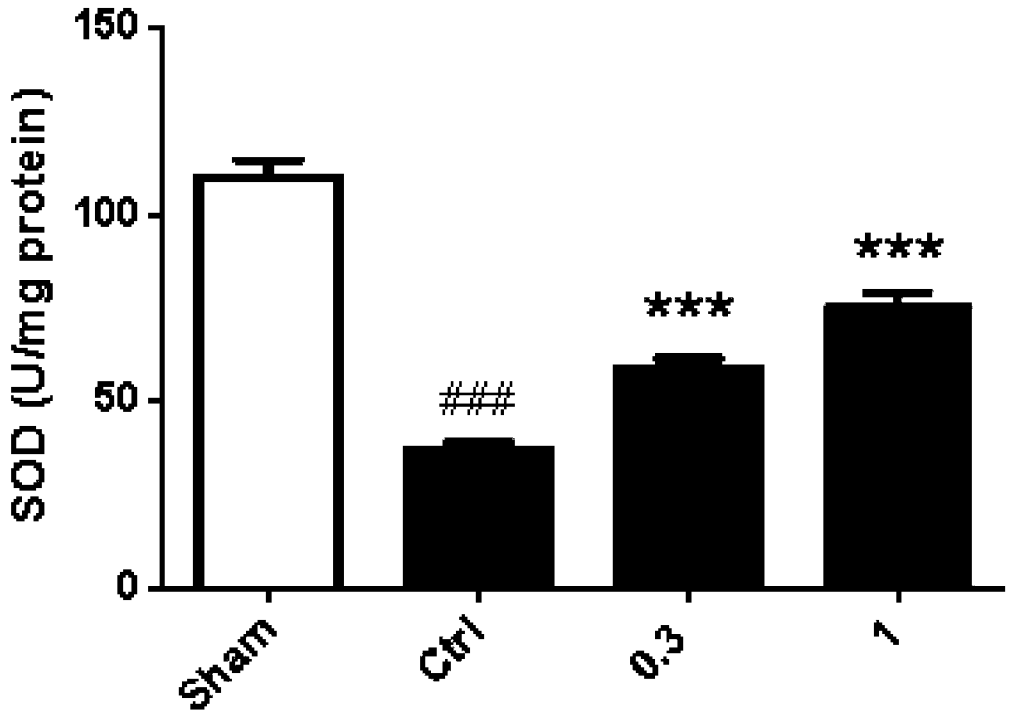


Figure 3.12 SOD specific activity of Tianma-treated rat brain tissue after MCAo. One unit of SOD was defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radicals. Data were expressed as mean ± SEM. $n \geq 7$. $^{###}p < 0.001$ as compared with sham group by Student's t-test, $***p < 0.001$ as compared with control by one-way ANOVA.

The GPx specific activity of the brain tissue from different groups was shown in Figure 3.13. The GPx specific activity of the sham, control, 0.3 g/Kg and 1 g/Kg Tianma-treated groups were 283.6 ± 17.5 nmol/mg protein, 196.1 ± 13.9 nmol/mg protein 219.6 ± 12.3 nmol/mg protein and 228.5 ± 8.9 nmol/mg protein, respectively. The GPx specific activity of the control group significantly decreased ($p < 0.01$) as compared with the sham group. The level of GPx specific activity increased in a dose-dependent mode after the Tianma treatment. However, no significant increase was observed in 0.3 g/Kg and 1 g/Kg Tianma-treated groups.

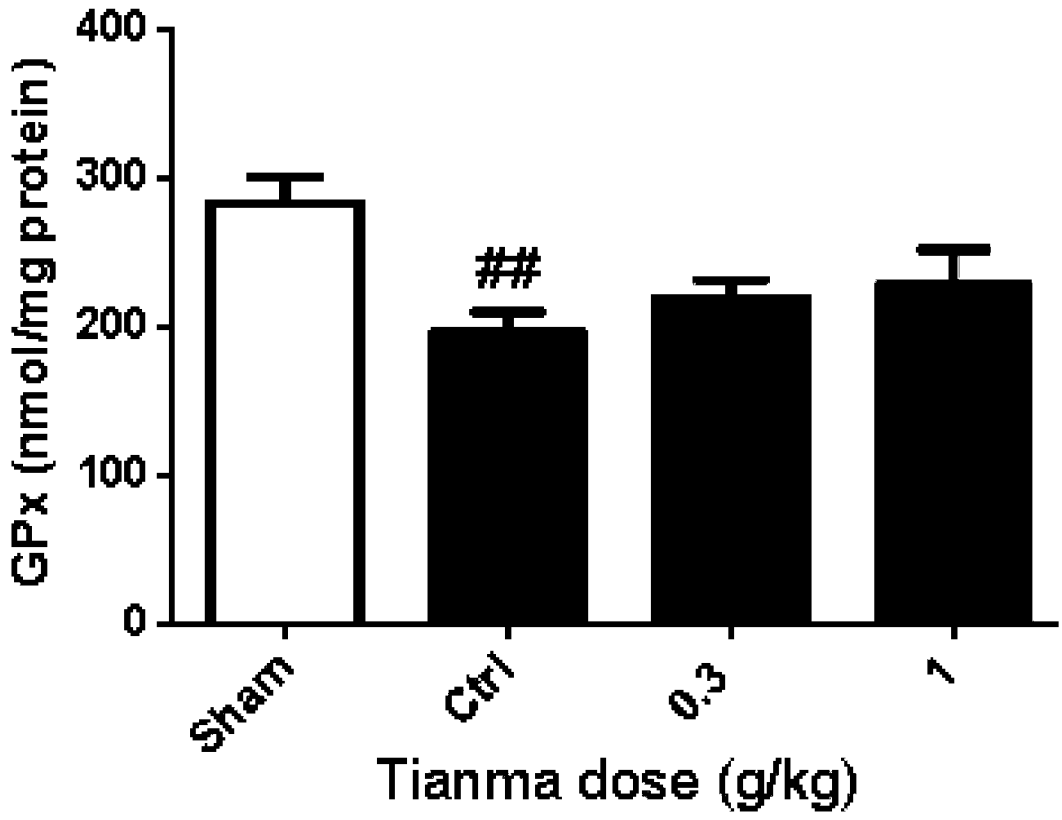


Figure 3.13 GPx specific activity of Tianma-treated rat brain tissue after MCAo. Data were expressed as mean ± SEM. $n \geq 7$. $^{\#\#}p < 0.01$ as compared with sham group by Student's t-test

The CAT specific activity of the brain tissue was shown in Figure 3.14. The CAT specific activity of the sham, control, 0.3 g/Kg and 1 g/Kg Tianma-treated groups were 24.4 ± 0.8 U/mg protein, 18.1 ± 0.3 U/mg protein, 20.3 ± 0.9 U/mg protein and 21.8 ± 0.6 U/mg protein, respectively. The CAT specific activity of the control group significantly decreased ($p < 0.001$) as compared with the sham group. A dose-dependent increase of the level of CAT specific activity was observed after Tianma treatment. Significant increases of CAT specific activity were observed in both 0.3 g/Kg ($p < 0.05$) and 1 g/Kg ($p < 0.001$) Tianma-treated groups.

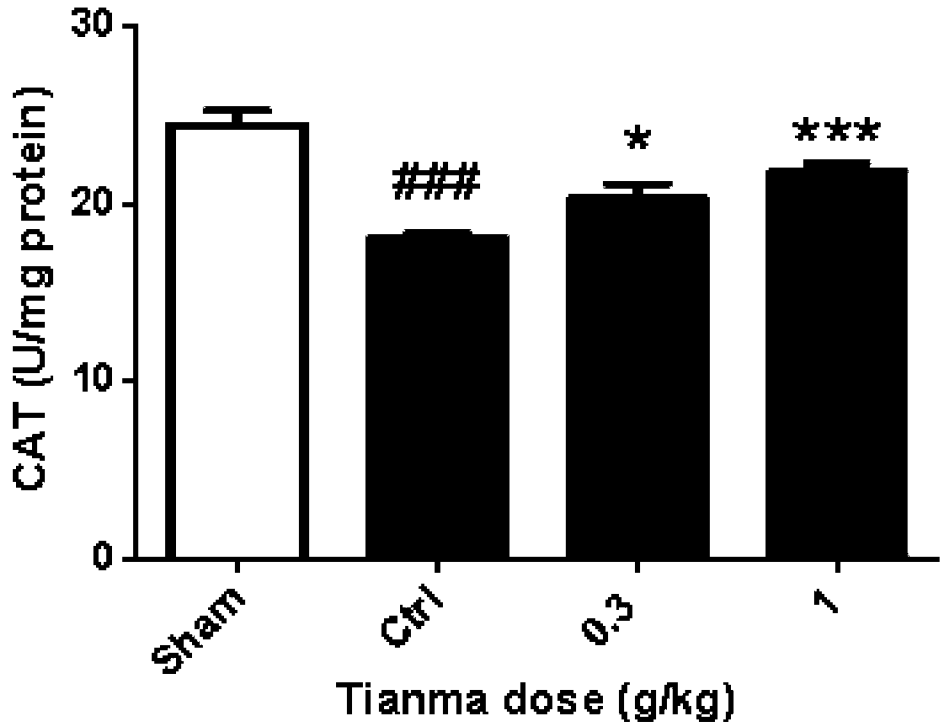


Figure 3.14 CAT specific activity of Tianma-treated rat brain tissue after MCAo. Data were expressed as mean ± SEM. n ≥ 7. $^{###}p<0.001$ as compared with sham group by Student's t-test, **p < 0.01, ***p < 0.001 as compared with control by one-way ANOVA.

3.3.3 Pro-inflammatory cytokines TNF-α and IL-6

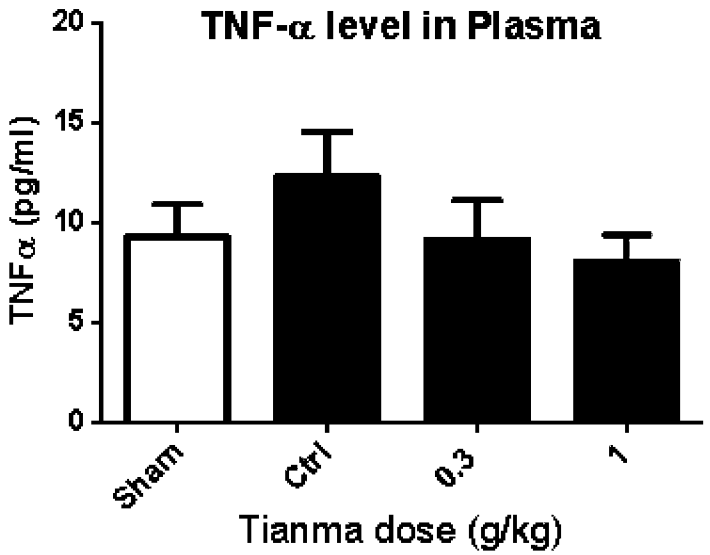
For measuring the anti-inflammatory effect of Tianma, both pro-inflammatory cytokines TNF-α and IL-6 in plasma and cerebrospinal fluid (CSF) were investigated by ELISA sets.

The plasma TNF-α concentration of the sham, control, 0.3 g/Kg and 1 g/Kg Tianma-treated groups were 9.3 ± 1.7 pg/mL, 12.3 ± 2.3 pg/mL, 9.2 ± 2.0 pg/mL and 8.1 ± 1.4 pg/mL, respectively as shown in Figure 3.15a. The concentration of TNF-α in plasma of the control group showed no significant increase when compared with the sham group. A dose-dependent decrease of TNF-α in plasma was observed after Tianma-treatment. The TNF-α level of 1 g/Kg Tianma-treated group was even lower than the sham group. However, no significant decrease in TNF-α was observed in 0.3 g/Kg and 1 g/Kg Tianma extract treated groups.

The concentration of TNF-α in CSF was also studied. However, most likely due to low TNF-α level and low sensitivity of the ELISA kit, no relevant result could be obtained.

The concentration of TNF-α in the brain homogenate was shown in Figure 3.15b. The TNF-α concentration in brain homogenate of the sham, control, 0.3 g/Kg and 1 g/Kg Tianma-treated groups were 126.1 ± 16.2 pg/mL, 233.0 ± 15.5 pg/mL, 176.3 ± 8.8 pg/mL, and 128.1 ± 11.7 pg/mL, respectively. TNF-α concentration in brain homogenate of the control group increased significantly ($p < 0.001$) as compared with the sham group. A dose-dependent decrease of TNF-α concentration in brain homogenate was observed after Tianma treatment. Significant decreases of the TNF-α concentration were observed in both 0.3 g/Kg ($p < 0.01$) and 1 g/Kg ($p < 0.001$) Tianma-treated groups

(a)



(b)

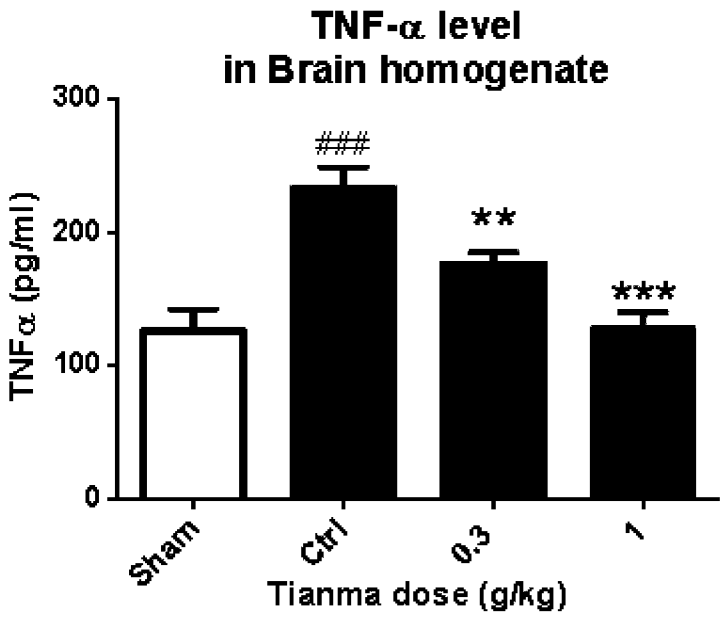


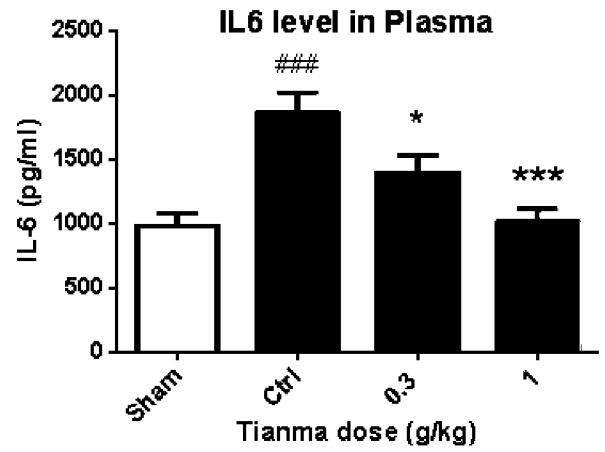
Figure 3.15 TNF-α concentration in Tianma-treated rat after MCAo in (a) plasma and, (b) brain homogenate. Data are expressed as mean ± SEM. n ≥ 7. $^{###}p<0.001$ as compared with sham group by Student's t-test, **p < 0.01, ***p < 0.001 as compared with control by one-way ANOVA.

IL-6 in plasma, CSF and brain homogenate were measured as shown in Figure 3.16a. The plasma IL-6 concentration in the sham, control, 0.3 g/Kg and 1 g/Kg Tianma-treated groups were 982.6 ± 99.9 pg/mL, 1865 ± 159.5 pg/mL, 1395 ± 136.7 pg/mL and 1016 ± 102.3 pg/mL, respectively. Plasma IL-6 concentration of the control group increased significantly ($p < 0.001$) as compared with the sham group. A dose-dependent decrease in plasma IL-6 was observed after Tianma treatment. Significant decreases of plasma IL-6 were observed in 0.3 g/Kg ($p < 0.05$) and 1 g/Kg ($p < 0.001$) Tianma-treated groups.

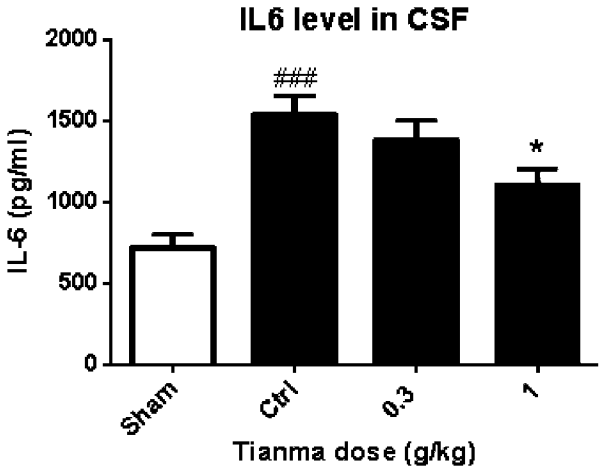
The concentration of IL-6 in CSF was shown in Figure 3.16b. The IL-6 concentration in CSF of the sham, control, 0.3 g/Kg and 1 g/Kg Tianma-treated groups were 720.8 ± 83.8 pg/mL, 1540 ± 116.1 pg/mL, 1385 ± 118.3 pg/mL and 1108 ± 100.2 pg/mL, respectively. A significant increase ($p < 0.001$) in IL-6 concentration in of control group was observed as compared with the sham group. The IL-6 concentration decreased dose-dependently after Tianma treatment. A significant decrease of the IL-6 was observed in 1 g/Kg ($p < 0.05$) Tianma-treated group.

The concentration of IL-6 in brain homogenate was shown in Figure 3.16c. The IL-6 concentration in brain homogenate of the sham, control, 0.3 g/Kg and 1 g/Kg Tianma extract treated groups were 4584 ± 166.0 pg/mL, 5286 ± 72.5 pg/mL, 4905 ± 125.9 pg/mL and 4626 ± 110.1 pg/mL, respectively. IL-6 concentration in brain homogenate of the control group increased significantly ($p < 0.001$) as compared with the sham group. A dose-dependent decrease of IL-6 concentration in brain homogenate was observed after Tianma treatment. Significant decreases of the IL-6 concentration were also observed in both 0.3 g/Kg ($p < 0.05$) and 1 g/Kg ($p < 0.001$) Tianma-treated groups.

(a)



(b)



(c)

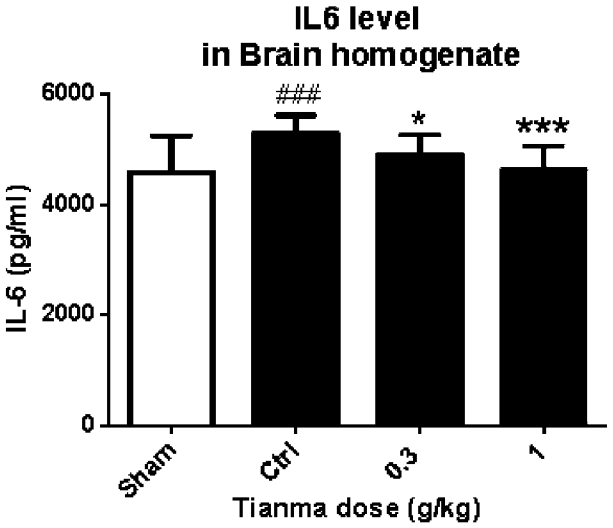


Figure 3.16 TNF-α concentration in Tianma-treated rat after MCAo in (a) plasma (b) CSF, and (c) brain homogenate. Data were expressed as mean ± SEM. n ≥ 7. ###p<0.001 as compared with sham group by Student's t-test, *p < 0.05, ***p < 0.001 as compared with control by one-way ANOVA.

3.4 Discussion

Due to the vast diversity in manifestations, causes, and anatomical sites in human ischemia stroke, which can only be controlled via animal studies (Molinari, 1988), *in vivo* studies was necessary for the investigation in ischemic stroke. Although up to this moment, none of existed stroke animal models (Ashwal and Pearce, 2001; De Lecinana *et al.*, 2010; Graham *et al.*, 2004) alone could completely mimic the human stroke due to the high variations of human clinical diseases. The use of stroke animal models, especially rodents (Li *et al.*, 2004), had a long history in the standardization of neurosensory and motor behaviors that facilitating the assessment of functional outcomes. This could provide possibilities of investigation of the mechanisms underlying ischemic stroke injury and the development of effective therapeutic solutions (Liu and McCullough, 2011).

There were numerous major rodent models of focal cerebral ischemia which have been developed (De Freitas *et al.*, 2008) and which could be further classified into (a) craniotomy not required, e.g. intra-arterial suture occlusion of the middle cerebral artery (MCAo) model (Longa *et al.*, 1989), embolic model (Busch *et al.*, 1997); (b) craniotomy required, e.g. Tamura's model (Tamura *et al.*, 1981); (c) cerebral venous thrombosis model (Schumacher, 1984) and (d) posterior cerebral circulation stroke model (Wojak *et al.*, 1991). The MCAo established by Kozuimi et al. (Koizumi *et al.*, 1986) in 1980s was a widely used cerebral ischemia rodent model for neuroprotective effect studies (Cui *et al.*, 2010; Kang *et al.*, 2006; Wang *et al.*, 2003). The extensively use on MCAo was due to the high incident rate of ischemic strokes in human occurred at MCA or its branches (De Freitas *et al.*, 2008). The middle cerebral artery occlusion in this model was achieved by insertion of an intraluminal filament from ECA into the ICA to occlude the origin of the MCA without craniectomy (Longa *et al.*, 1989; Schmid-Elsaesser *et al.*, 1998). The CBF at

MCA could be monitored by a laser-Doppler flowmetry, which is capable of penetrating the cranial bone for detection as the laser is scattered by moving hemoglobins resulting in a Doppler frequency shift (Dirnagl *et al.*, 1989). Reperfusion phase proceeded simply by retracting the filament from the vessel.

The model, such as the coating material of suture and location for the insertion of suture, was further modified in order to reduce subarachnoid hemorrhage and premature reperfusion (Belayev *et al.*, 1996; Longa *et al.*, 1989; Schmid-Elsaesser *et al.*, 1998). This model required a simpler and reduced invasive surgical procedure when compared with craniotomy model and lowered the risk of death due to complication. Furthermore, the infarcts induced by this model were consistent in almost all cases. Primary ischemic cell death could be found at striatum and overlying temporal, parietal, frontal cortex etc. (Garcia *et al.*, 1995; Kanemitsu *et al.*, 2002; Williams *et al.*, 2004), although there were small variations of infarcts at the thalamus, substantia nigra, and hypothalamus, etc. (Carmichael, 2005; Liu *et al.*, 2009). A clear ischemic core and penumbra could be easily identified in MCAo (Kaufmann *et al.*, 1999). Moreover, the survival of the animals after the surgery could last for several months to allow long-term investigation of stroke recovery and rehabilitation (Graham *et al.*, 2004).

Neurological score was a quantified measurement of the neurological deficit as a result of ischemia. The location of MCA was close to the motor cortex on the posterior frontal lobe (Carmichael, 2005). MCAo would cause ischemia in that brain area and resulted in hemiparalysis (Bederson *et al.*, 1986b). From the result of neurological score (Figure 3.10), the average score of the control group was 2.64, which was approximately 3 according to the 4-point scale (Table 3.1). This indicated the MCAo would induce neurological impairment to operated rats (Bederson *et al.*, 1986b). The average neurological scores of 0.3 g/Kg and 1 g/Kg Tianma-treated

group ameliorated from 2.64 to 2.40 and 1.85, respectively when compared with the control group. This showed that the Tianma was able to improve neurological deficit after ischemia. There would be a potential use of Tianma for stroke patients during rehabilitation.

2,3,5-Triphenyltetrazolium hydrochloride, known as TTC, was originally used to test the viability of seeds (Lillie and Conn, 1969). TTC had been used as a stain for the detection of ischemic infarct in mammalian tissue in 1958 (Bederson *et al.*, 1986a). TTC could directly indicate neuronal cell survival as TTC could be reduced by dehydrogenases in the mitochondria of living cells to developed a deep red, fat soluble, light-sensitive compound (formazan). The normal brain tissue would be turned deep red but not the infarct area. Thus the infarct area could be distinguished as the white area remained (Figure 3.6) (Bederson *et al.*, 1986a; Lillie and Conn, 1969) for quantification (Zhan and Yang, 2006a). From the TTC staining (Figure 3.11a), the white region in the brain slices from the control, 0.3 g/Kg and 1 g/Kg Tianma-treated group indicated that the brain infarct was successfully induced by MCAo.

From the results of average infarct volume (Figure 3.11b, 3.11c), significant reduction in brain infarct was shown in both 0.3 g/Kg and 1 g/Kg Tianma-treated group in a dose-dependent manner. This result was consistent with that of the neurological score. As the average infarct volume reflected the amount of damaged neuronal cells, reduction in the average infarct volume implied that more neuronal cells were protected by the Tianma treatment. This could explain the improvement of the neurological deficit.

The findings in the average neurological scores and average infarct volume correlated with previous studies on Tianma active ingredients, such as Gastrodin (GAS) (Zeng *et al.*, 2006) and 4-Hydroxybenzyl alcohol (4-HBA) (Yu *et al.*, 2011) or organic extract (Tsai *et al.*, 2011) which asserted neuroprotective effects on ischemic

stroke in MCAo model. For the study on GAS, 99.6% pure 50 mg/Kg and 100 mg/Kg GAS were used. The 4-point neurological scoring system (Longa *et al.*, 1989) was very similar to the one we used in this study. The scores of 50 mg/Kg and 100 mg/Kg GAS reduced to neurological scores of 1.9 and 1.7 respectively from score of 2.2 in the control group. The percentage of neurological score reduction in 50 mg/Kg and 100 mg/Kg Gastrodin group were 30% and 35% respectively. As we have quantified the amount of Gastrodin in our Tianma water extract, which was 2.87% v/v. 0.3 g/Kg and 1 g/Kg Tianma extract in our studies contain 8.61 mg/Kg and 28.7 mg/Kg Gastrodin respectively only, but resulted in 9.09% and 30% neurological score reduction. This finding might be due to other active compounds in Tianma extract and the synergistic effect among various compounds in Tianma water extract, such as GAS and 4-Hydroxybenzaldehyde (4-HBAL), etc.

Free radicals are produced all the time in our bodies. Under normal condition, detoxification of these radicals would be processed by glutathione, ascorbic acid and Vitamin E. However, reoxygenation during reperfusion would lead to formation of superoxide and nitric oxide. Anti-oxidative enzymes including SOD, GPx and CAT would be the major scavengers for these free radicals (Chan, 1996). Low concentrations of these enzymes would cause excessive ischemia and reperfusion-induced oxygen free radicals retained in the brain (Mizuno and Ohta, 1986) and led to brain dysfunction and neuronal cell death (Braughler and Hall, 1989; Oliver *et al.*, 1990). The restoration of anti-oxidative enzyme activities would improve neuronal cells survival during ischemia-reperfusion (Luo *et al.*, 2004b; Shukla *et al.*, 2008; Zhan and Yang, 2006a). It is of great interest to investigate the regulation of anti-oxidative enzymes by Tianma in protecting the brain after stroke.

The ischemia-reperfusion injury could be reduced by the SOD as it can scavenge superoxide (McCord, 1985). It was well-stated in many studies that the

SOD activity would be upregulated after ischemia-reperfusion, in providing neuroprotective effects (Luo *et al.*, 2004a; Shukla *et al.*, 2008; Zhan and Yang, 2006b). The disproportionation of superoxide was catalyzed by SOD into oxygen and hydrogen peroxide (Albrecht-Gary *et al.*, 1998; Fridovich, 1989). There were three types of SODs found in neuronal cells, classified according to their metal ion requirements and the anatomical distribution (Chan, 1996), including CuZn-SOD (SOD 1) in cytoplasm (Chan, 2001), Mn-SOD (SOD 2) in mitochondrial matrix and the inner membrane as well as EC-SOD (SOD 3) in the extracellular space, cerebrospinal fluid, and cerebral vessels (Marklund, 1982; Warner *et al.*, 2004).

GPx was important for neuroprotection as it could catalyze the conversion of hydroperoxides, such as hydrogen peroxide to water and glutathione disulfide (GS-SG) by glutathione (GSH) co-factors (Bhabak and Mugesh, 2010; Warner *et al.*, 2004) which played a role in redox defense (Flohe *et al.*, 1973; Rotruck *et al.*, 1973). GPx was one of the selenoproteins which could be further classified into 6 groups which localized differently. GPx1 and GPx4 were the most abundant GPx expressed in brain, including neurons and astrocytes (Schweizer *et al.*, 2004). It was reported that GPx1 was closely related to the neuroprotection in patients with Parkinson's disease and dementia with Lewy bodies (DLB) as GPx1 acted as an anti-oxidative enzymes for neuronal defenses (Power and Blumbergs, 2009). The neuroprotective effects of GPxs via anti-oxidation during ischemia-reperfusionhad been proven in various studies (Luo *et al.*, 2004b; Power and Blumbergs, 2009; Qu *et al.*, 2009; Saleem *et al.*, 2006).

CAT, widely found in animals, plants, fungi and bacteria and localized in the peroxisomes of cells (Warner *et al.*, 2004), played a protective role during ischemia-reperfusion injury as a hydrogen peroxide scavenger (McCord, 1985). It could catalyze the conversion of hydrogen peroxide to harmless water and oxygen

(Chelikani *et al.*, 2004). CAT had been reported in different studies of neuroprotection (Ambikar *et al.*, 2010; Kuo *et al.*, 2011; Peng *et al.*, 2005). During ischemia-reperfusion, the downregulated CAT activity would reduce neuroprotection (de la Torre, Ma Rosario *et al.*, 1996; Margaill *et al.*, 2005).

From the result of anti-oxidative enzymes, SOD, GPx and CAT (Figure 3.12-3.14), their activities were significantly downregulated in the control group after MCAo as compared to the sham group. These results confirmed with other similar studies on the anti-oxidative activities after MCAo (Yamato *et al.*, 2011; Zhang *et al.*, 2006; Zhou *et al.*, 2006). After Tianma treatment, dose-dependent increases of enzyme activities were observed in three enzymes. The increased anti-oxidative enzyme activities were consistent with the improvement of the neurological score and brain infarct volume. These results together suggested that 1 hour pretreatment with Tianma water extract could improve the MCAo induced cerebral ischemia–reperfusion injury in rats due to its anti-oxidative properties.

From a previous study on 4-HBA, a known neuroprotective agent of anti-oxidative effect (Yu *et al.*, 2010), the SOD activity in MCAo operated rats after the 4-HBA had increased by about 60% (Yu *et al.*, 2011) when compared with control. In our study, the 1 g/Kg treatment of Tianma water extract could almost double the SOD activity after MCAo. This drastic increase of SOD activity suggested that the Tianma water extract has a potent anti-oxidative effect. Comparisons of GPx and CAT activities could not be made due to limited works on the active ingredients of Tianma.

During ischemia, gliosis, a nonspecific reactive activation, proliferation and hypertrophy of macrophages and microglia would resulte in response to brain tissue damage (Perry and Gordon, 1991). This is originally a defense mechanism for repair, restoration of blood supply by means of alteration of blood brain barrier integrity

(Norton *et al.*, 1992; O'Callaghan, 1991). However, the interactions between inflammation and glial cell activities were close to each other as glial cells were responsible for the production of cytokines which could activate the glial cells in return (Eng, 1988; Sawada *et al.*, 1989). More neutrophils would migrate to the infarct area and further intensified the inflammation (Suk, 2007). The gliosis could be induced by TNF-α and IL-6 (Balasingam *et al.*, 1994). The expressions of these two pro-inflammation cytokines were associated with the degree of brain injury (Feuerstein *et al.*, 1998).

During cerebral ischemia, TNF-α was upregulated (RW.ERROR - Unable to find reference:344; Liu *et al.*, 1994) which would induce larger excitotoxicity brain lesions in ischemia (Dommergues *et al.*, 2000). From clinical studies, volume of brain infarct and ischemic stroke severity were associated with elevated TNF-α level (Yang *et al.*, 1998; Zaremba and Losy, 2001). Increased TNF-α level after MCAo surgery would exacerbate edema and ischemic injury in animal studies (Adibhatla and Hatcher, 2007; Caso *et al.*, 2006; Vakili *et al.*, 2011). Moreover, both apoptosis and necrosis could be induced by intracellular signaling pathways (Reid *et al.*, 1989).

The IL-6 expression was elevated in MCAo-operated rats (Clark *et al.*, 1999; Loddick *et al.*, 1998) and similar observation was reported in acute stroke patients (Basic Kes *et al.*, 2008; Tarkowski *et al.*, 1995) that the IL-6 level correlated to the brain infarct volume or stroke severity (Fassbender *et al.*, 1994; Perini *et al.*, 2001). The inflammatory injury or infarct pathogenesis might be contributed by IL-6 as it was known as having the pro-inflammatory effects (Brett *et al.*, 1995; Johnson *et al.*, 1998; Ulich *et al.*, 1989). Furthermore, it had been reported that IL-6 could be upregulated by TNF-α (Shalaby *et al.*, 1989). It seemed there was a correlation between IL-6 and cerebral ischemia, but no definite answer to the role played by IL-6 could be drawn (Clark *et al.*, 2000; Matsuda *et al.*, 1996; Smith *et al.*, 2004).

From our studies (Figure 3.15 and 3.16), the TNF-α and IL-6 levels, except TNF-α concentration in plasma, were increased significantly when compared with sham group. These findings correlated well with previous studies (Clark *et al.*, 1999; Loddick *et al.*, 1998; Vakili *et al.*, 2011). After Tianma treatment, dose-dependent decreases in the TNF-α and IL-6 levels among plasma, CSF and brain homogenate were observed. However, only negative signals were received in the CSF samples as compared to the standard. We suggested that it was most likely due to low sensitivity of the kit as well as the limited volume of CSF. These findings showed that besides the phenolic compounds from methanolic extracts of Tianma (Jang *et al.*, 2010; Lee *et al.*, 2006) which demostrated anti-inflammatory effects, this property was in the water extract as well, leading to downregulation of pro-inflammatory cytokines.

In conclusion, Tianma extract showed its neuroprotective effects on *in vivo* MCAo, which included reduction of neurological deficits and brain infarct volume. There might be due to Tianma extract having anti-oxidative effect by upregulating the anti-oxidative enzymes SOD, GPx and CAT as well as anti-inflammatory effect by downregulating pro-inflammatory cytokines TNF-α and IL-6. Further *in vitro* studies would be performed to verify the underlying mechanisms of Tianma extract on protection against ischemic stroke (See section 4).

Chapter 4 *In vitro* study on anti-oxidative, anti-inflammatory effects of Tianma extract on ischemic stroke

4.1 Introduction

We found that Tianma extract gave promising neuroprotective effects on regulating anti-oxidative enzymes as well as pro-inflammatory cytokines in the MCAo model (see Chapter 3). However, the cellular mechanisms underneath these findings are unclear. From clinical studies, anti-oxidant activities in patients were diminished after ischemic stroke (Cherubini *et al.*, 2000) and ischemic brain injury was partly contributed by post-ischaemic inflammation (Feuerstein *et al.*, 1998). Among all mechanisms of cell injuries (See section 1.1.3) during ischemic stroke, we chose to focus on oxidative stress due to production of free radicals and inflammation. As mentioned, reduction of neuronal cell death is one of the western conventional treatment focused by various groups (Ferriero, 2004; Moskowitz *et al.*, 2010), but no drugs for stroke treatment targeting on oxidative and inflammatory pathways gained USFDA approval as therapeutic agent for acute ischemic stroke treatment yet (Roach *et al.*, 2010).

Ethanolic extract and diethyl ether fractions of Tianma were proven to be an anti-oxidant and free radical scavenger for OH・, respectively (Kim *et al.*, 2003; Liu and Mori, 1992). In addition, Tianma ethanolic extract also inhibited NO production and downregulated the inflammatory effectors such as iNOS and COX-2 expression (Ahn *et al.*, 2007). However, none of these studies were on anti-oxidative and anti-inflammatory effects of Tianma water extract. In this chapter, we investigated whether there were any similar effects by Tianma water extract using oxidative stress model, H_2O_2-induced oxidative stress model and oxygen-glucose deprivation (OGD) model using rat PC 12 cells. For inflammation mechanistic study, lipopolysaccharide (LPS)-induced inflammation was performed using murine RAW 264.7 macrophages.

4.2 Materials and methods

4.2.1 Anti-oxidation

4.2.2.1 Rat pheochromocytoma PC 12 cells

Rat pheochromocytoma (PC 12) cells (Figure 4.1) were purchased from American Type Culture Collection (ATCC) (Manassa, VA, USA) and were maintained at subconfluent density 80% in RPMI-1640 (Gibco, Life Technologies, California, USA) medium supplemented with 10% v/v heat-inactivated horse serum (HS), 5% v/v fetal bovine serum (FBS), 1% v/v Penicillin Streptomycin (Pen Strep) according to ATCC's protocol. All HS, FBS and Pen Strep were purchased from Gibco (Life Technologies, California, USA). The cells were cultured in 150 cm^2, poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) coated tissue culture flasks in humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

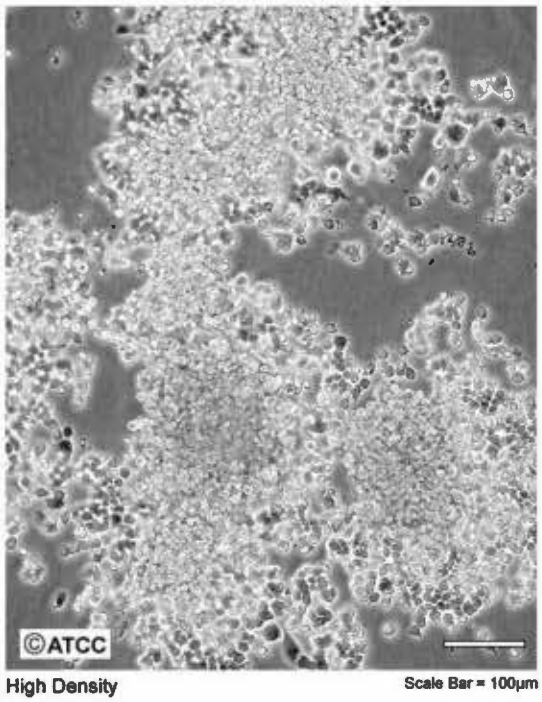


Figure 4.1 The PC 12 cells morphology in high density (adopted from ATCC)

4.2.2.2 H_2O_2-induced oxidative stress model

As we had to select a suitable H_2O_2 concentration to induce oxidative stress with about 50% cell death, as well as to investigate Tianma extract toxicity on PC 12 cells, MTT assay was performed to assess the cell survival. PC 12 cells of $1x10^5$ cells/well were initially seeded on 96-well plates which were precoated with poly-L-lysine and was incubated overnight. H_2O_2 with concentration ranged from 0 to 800 μM or Tianma extract with concentration ranged from 0 to 1000 μg/mL were incubated with PC 12 cells in different plates for 24 hours in humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The viability of cell was determined by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay.

75 μM H_2O_2 was selected as the concentration of H_2O_2-induced oxidative stress on PC 12 viability after precondtioning. PC 12 cells were seeded overnight. The cells were treated with 8 different concentrations of Tianma extract, range from 0 to 700 mg/mL for 24 hours in the presence or absence of 75 μM H_2O_2. MTT assay was performed at the end of the treatment.

4.2.2.3 Oxygen glucose deprivation (OGD) reperfusion model

PC 12 cells were plated at $1x10^5$ cells/well in 96-well plates precoated with poly-L-lysine overnight in complete RPMI 1640 culture medium with 10% v/v horse serum, 5% v/v FBS and 1% v/v Pen Strep at 37°C with 5% CO_2 and 95% air. As we had to know effects of different OGD durations on PC 12 cell survival, we referred to two commonly used ischemic duration, 8 or 16 hours (Kritis *et al.*, 2011; Xu *et al.*, 2000) and assessed the cell survival after OGD using MTT assay. Cells were washed twice in PBS and switched to glucose free RPMI 1640 medium (Gibco, Life Technologies, California, USA) supplemented with 2% v/v horse serum, 1% v/v FBS

and 1% v/v Pen Strep, known as OGD medium. The plate was then relocated to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) (Figure 4.2). The chamber was flushed with a gas mixture of 95% N_2 and 5% CO_2 for 30 mins at room temperature at air flow of 3 L/min. After flushing, the chambers were sealed and incubated at 37 °C for 16 or 8 hours and followed by reperfusion. OGD medium was placed with reperfusion medium, which is glucose-containing RPMI 1640 culture medium but with only 2% v/v horse serum, 1% v/v FBS and 1% v/v Pen Strep. MTT assay was performed to determine the cell viability at the end of the experiment for selecting the OGD duration, depending on which resulted in a lower cell survival after the treatment.

Figure 4.2 The modular hypoxia incubation chamber (Adopted from manufacturer's website)

For investigating of the effect of Tianma extract on the survival of PC 12 under OGD-induced oxidative stress, we used the same procedures as investigating the effects of different duration until the replacement by OGD medium. After washing twice by PBS, cells were supplemented with OGD medium at the presence or absence of 6 different concentrations of Tianma extract (0, 62.5, 125, 250, 500, 1000 μg/mL). The plate was then relocated to a modular incubator chamber and flushed as described above. After flushing, the chambers were sealed and incubated at 37°C for 16 hours.

For the normal plate, cells were washed with PBS twice after overnight incubation. Cells were supplemented with the medium which has the same composition as the reperfusion medium mentioned before. The normal plate was also incubated for 16 hours with 5% CO_2 and 95% air at 37°C but without being flushed with 95% N_2 and 5% CO_2 or sealed in the modular hypoxia incubation chamber.

Both OGD and normal plates were proceeded to the reperfusion after 16 hours of OGD or normal incubation. The medium of all plates were replaced by reperfusion medium instead. Both underwent reperfusion for 24 hours in humidified atmosphere of 5% CO_2 and 95% air at 37 °C and followed by MTT assay.

4.2.2.4 MTT cell proliferation assay

MTT assay was used to investigate cell proliferation or simply cell viability. 5 mg/mL of MTT solution was prepared by dissolving MTT powder in PBS, facilitated by sonication. MTT solution was filtrated before use to prevent any impurities. MTT solution was diluted in medium used in the assay in v/v ratio 1:10 before adding to the cells at the end of the treatments. After 2 hours MTT incubation, the reaction was terminated by the addition of DMSO to the cell culture 2 hours after MTT addition. The absorbance was measured at 540 nm using uQuant microplate

spectrophotometer (BioTek Instruments, Winooski, VT, USA).

4.2.2.5 Measurement of reactive oxygen species (ROS) activity by 2',7' dichlorofluorescein diacetate (DCFDA)

The DCFDA fluorescent signal was directly proportional to intracellular ROS levels (Nemoto and Finkel, 2002; Sponne *et al.*, 2003). 50 μM DCFDA (Invitrogen Corporation, Carlsbad, CA, USA) was prepared by dissolving DCFDA in DMSO. Final concentration of 10μM DCFDA was added to OGD and normal plates at the end of experiment. The plates were incubated at humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 1 hour. The fluorescent signal was then measured by a fluorescent microplate reader (FLUOstar OPTIMA, BMG LABTECH, Germany) with software (Fluoster Optima software Version 1.30-0, BMG LABTECH GmbH, Germany), measured by excitation at 488 nm and emission at 530 nm.

The fluorescent signal of the cells was captured under the inverted research fluorescence microscope (Olympus IX71, Japan) and SPOT camera (Spot Imaging Solutions, Diagnostic Instruments, Inc., Michigan, USA).

4.2.2 Anti-inflammation

4.2.2.1 Mouse leukaemic monocyte macrophage RAW 264.7 cells

The mouse leukaemic monocyte macrophage RAW 264.7 cell line (Figure 4.3) was purchased from ATCC and cultured at subconfluent density in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Manassas, VA, USA) supplemented with 10% v/v FBS and 1% v/v Pen Strep. The cells were cultured in humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

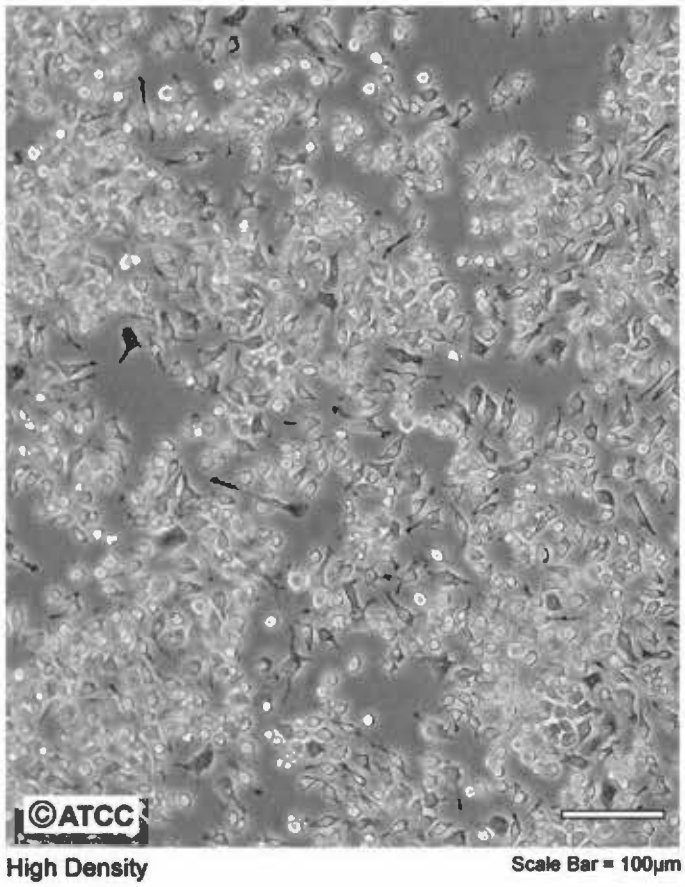


Figure 4.3 The RAW 264.7 cells morphology in high density (adopted from ATCC)

4.2.2.2 Lipopolysaccharide (LPS)-induced inflammation and nitric oxide (NO) production assay

Murine RAW 264.7 macrophages were seeded (4×10^5 cells/well) at 24-well plate with DMEM supplement overnight. The cells were treated with 4 different concentraions of Tianma extract (125, 250, 500, 1000 mg/mL) at 1 mL/well for 24 hours in the presence or absence of 1 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for the induction of inflammation. NO production which is an indicator of inflammation.

The cytotoxicitiy of Tianma extract and LPS were assessed by MTT assay, followed by the measurement of NO production. MTT assay procedures were the same as that for PC 12 cells (see section 4.2.2.4). The production of NO was reflected by the accumulation of nitrite, which was a stable product of NO in the culture medium. Equal amount (100 μL) of Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) and cell free medium were incubated at room temperature for 15 mins. The amount of nitrite produced was quantified by a uQuant microplate spectrophotometer (absorbance at 540nm) using sodium nitrite as standard.

4.2.2.3 Measurement of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β by ELISA sets

BD OptEIA Enzyme-linked immunosorbent assays (ELISA) (BD Biosciecnces, San Diego, CA, USA) sets were used to measure pro-inflammatory cytokines TNF-α, IL-6 and IL-1β. RAW 264.7 macrophages were seeded and treated in a way similar to NO production assay (see section 4.2.2.2). The cell free supernatent was collected and used for measuring the pro-inflammatory cytokines byELISA.

The ELISA sets were used according to manufacturer's instruction. The

protocol was summarized as follow. The capture antibodies of TNF-α, IL-6 and IL-1β were reconstituted in coating buffer as stated by the manufacturer. The plates coated with capture antibodies and were sealed. The wells were aspirated with wash buffer after overnight incubation at 4 ^{o}C. The wells were then soaked with assay diluent which was 10% FBS in PBS for 1 hour at room temperature. The wells were aspirated with wash buffer again to remove the assay diluent.

Standard was diluted in assay diluent in 2-fold dilution for a total of 6 concentration gradients for plotting the standard curve. The highest standard concentrations for TNF-α, IL-6 and IL-1β were the same, 500 pg/mL. 100 μL of each sample and standards were added to each well. The plates were sealed and incubated for 2 hours at room temperature. After incubation, the wells were aspirated and washed with wash buffer. 100 μL of detection antibody, diluted in assay diluent for TNF-α, IL-6 and IL-1β, were added accordingly and incubated for 1 hour at room temperature. The wells were aspirated and washed with wash buffer again. 100 μL of diluted enzyme reagent (Streptavidin-HRP) was added to each well and incubated for 30 mins at room temperature. The wells were aspirated and washed with wash buffer thoroughly after incubation. TMB Substrate Reagent A and B were mixed in 1:1 v/v ratio to give TMB Substrate Solution. 100 μL of TMB Substrate Solution was added to each well and further incubated for 30 mins in the dark at room temperature. 50 μL of stop solution (2N H_2SO_4) was added to each well to stop the reaction. The absorbance at 450 nm was measured by the uQuant microplate spectrophotometer within 30 minutes.

4.2.2.4 Determination of inflammatory factors inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression by Western blotting

Whole cell extracts were prepared by lysing the cells with RIPA lysis buffer

(150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, protease and phosphatase inhibitor cocktails). In order to normalize the protein expression by using same amount of protein in samples, protein concentration of each sample was quantified by BCA assay as described in section 3.2.5.2.

Equal amount of whole cell protein extract was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then transferred onto a polyvinlylidene difluoride (PVDF) membrane (Amersham Hybond™-P, GE Healthcare, Piscataway, NJ, USA). The transblotted membranes were blocked with 0.5% non-fat milk in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 1 hour. The membranes were incubated overnight at 4 °C with specific antibodies against iNOS, COX-2 (BD Biosciences, San Diego, CA, USA) and β-actin (Cell Signaling Technology, Danvers, MA, USA). The blots were then washed three times with TBST before incubating with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) for 2 hours. The blots were then washed for another three times with TBST before developing. The blots were developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ, USA) and detected with ChemiDoc XRS+ system (Bio-rad, Hercules, CA, USA). Images were processed and quantified with Image Lab 3.0 (Bio-rad, Hercules, CA, USA).

4.2.2.5 Statistical Analyses

The differences between the control and normal groups were tested with unpaired student t-test unless specified. The differences between the control groups and different dose treatment groups were tested with one-way analysis of variance (one-way ANOVA) by Dunnett's multiple comparisons test unless specified. All

statistical analyses were performed at 5% level of significance ($p<0.05$) by using GraphPad Prism software version 6.01. Data were expressed as mean ± standard deviation (SD) in all *in vitro* experiments.

4.3 Result

4.3.1 Anti-oxidation

4.3.1.1 MTT Cell survival assay of H_2O_2-induced oxidative stres measured by MTT assay

Cell survival after treating with different concentration of H_2O_2 and Tianma extract, as well as those after co-treatment of H_2O_2 and Tianma extract on PC 12 cells was assessed by the MTT assay. For cell survival after H_2O_2 treatment of 0, 25, 50, 100, 200, 400, 800 μM were 100 ± 10.8%, 100 ± 11%, 72.8 ± 8.0%, 15.5 ± 5.3%, 1.5 ± 2.3%, 0.9 ± 1.1% and 1.2 ± 2.0%, respectively. $H_2O_2 \geq 50\mu M$ showed a significant decrease ($p < 0.001$) of cell survival as compared with that of control (0 μM H_2O_2) (Figure 4.4a).

Cell survival after Tianma treatment of 0, 125, 250, 500, 1000 μg/mL were 100 ± 11.3%, 116 ± 16.3%, 115 ± 18%, 125 ± 13.5, 127 ± 20.9%, respectively. Tianma concentration ≥ 125 μg/mL showed a significant increase ($p < 0.05$ for 125, 250 μg/mL ; $p < 0.001$ for 500, 1000 μg/mL) in cell survival as compared with that of control (Figure 4.4b).

Based on result from cell survival after treatment with different concentration of H_2O_2, 75 μM H_2O_2 was selected for the induction of oxidative stress. Cell survival of normal, 0, 100, 200, 300, 400, 500, 600, 700 μg/mL were 100 ± 12.7%, 59.9 ± 16.1%, 63.6 ± 16.7%, 73.4 ± 9.7%, 83.8 ± 12.3%, 85.9 ± 15.1%, 82.2 ± 12%, 83.1 ± 12.6%, 93.6 ± 20.9%, respectively (Figure 4.4c). The presence of 75 μM H_2O_2 significantly reduced ($p < 0.001$) the cell survival when compared with that of normal. Tianma extract ≥ 300 μg/mL significantly increased ($p < 0.001$, except $p < 0.01$ for 500 μg/mL) the cell survival as compared with that of control (0 μg/mL Tianma extract).

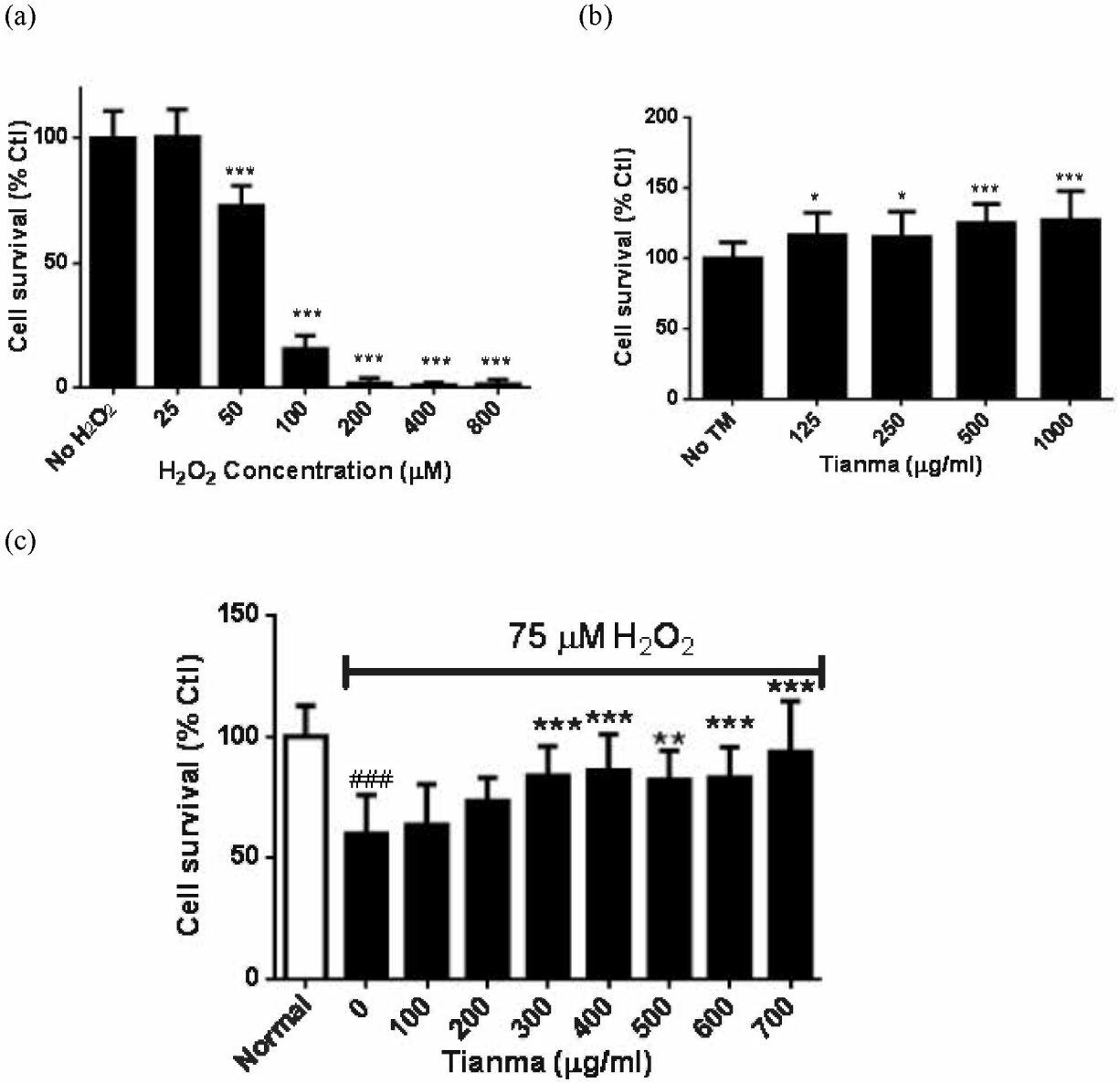


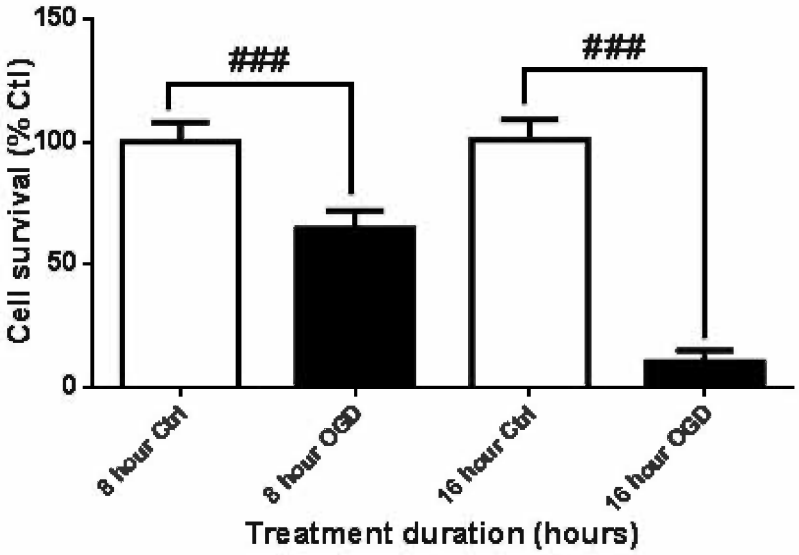
Figure 4.4 H_2O_2-induced oxidative stress on PC 12 cells survival. (a) H_2O_2-induced cytotoxicity on PC 12 cells. (b) Tianma cytotoxicity on PC 12 cells. (c) The cell survival after co-treatment of Tianma extract with the presence or absence of 75 μM H_2O_2 on PC 12 cells. Data were expressed as mean ± SD, n = 18 in 3 individual experiments. $^{###}p < 0.001$ as compared with normal group by Student's t-test. *p < 0.05 , **p < 0.01, ***p < 0.001 as compared with control by one-way ANOVA.

4.3.1.2 Cell survival of OGD-reperfusion model measured by MTT

PC 12 cell survival after treated with different duration of ischemia and anti-oxidative effect of Tianma extract in OGD-reperfusion model were assessed by the MTT assay. The results were shown in Figure 4.5. Cell survival for different duration (8 hours or 16 hours) of ischemia with the normal medium or OGD medium, followed by 24 hours of reperfusion in normal medium were 100 ± 7.8% (8 hours + normal medium), 64.6 ± 7.4% (8 hours + OGD medium); 101 ± 8.3% (16 hours + normal medium), 10.5 ± 4.6% (16 hours + OGD medium), respectively (Figure 4.5a).

Based on the cell survival after different ischemic duration, 16 hours ischemia was selected for OGD. The cell survival with or without 16 hours ischemia and followed by 24 hours reperfusion on PC 12 cells was measured by MTT. The cell survival of normal, 0, 62.5, 125, 250, 500, 1000 μg/mL were 99.3 ± 9.0%, 11.6 ± 7.4%, 27.5 ± 11.1%, 47.3 ± 4.6%, 54.9 ± 8.2%, 70.8 ± 8.3%, 70.3 ± 7.3%, respectively. The OGD could significantly reduce ($p < 0.001$) the cell survival when compared with that of normal. Concentration ≥ 62.5 μg/mL significantly increased ($p < 0.001$) the cell survival when compared with control.

(a)



(b)

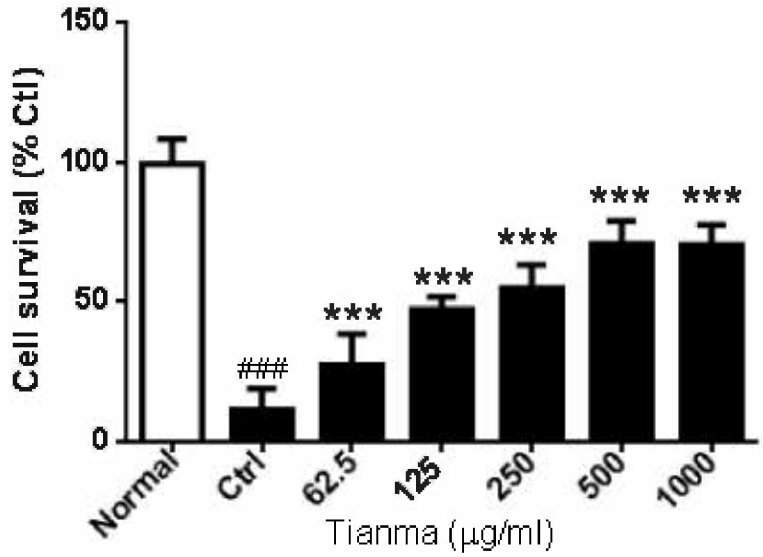


Figure 4.5 OGD-induced oxidative stress on PC 12 cells survival (a) Effect of different OGD durations on PC 12 cell survival (b) The cell survival after Tianma treatment with the presence or absence of 16 hours ischemia and followed by 24 hours reperfusion on PC 12 cells. Data were expressed as mean ± SD, n = 18 in 3 individual experiments. $^{###}p < 0.001$ as compared with normal group by Student's t-test. ***$p < 0.001$ as compared with control by one-way ANOVA.

4.3.1.3 Measurement of reactive oxygen species (ROS) activity by DCFDA

The effects of different concentration Tianma extract on ROS production in PC 12 cells after 16 hours of ischemia were assessed by the DCFDA fluorescent signal. Significant ROS production was induced by OGD as shown in Figure 4.6a. The DCFDA signal of normal and OGD-treated group were 8690 ± 1100 units and 13791 ± 987 units, respectively. There was a significant increase ($p < 0.001$) of DCFDA signal in the OGD-treated group when compared with the normal group.

The DCFDA signal Tianma-treated groups in OGD with concentration of 0, 62.5, 125, 250, 500, 1000 μg/mL were 99.6 ± 6.6%, 83.3 ± 11.7%, 75.6 ± 14.8%, 70.1 ± 9.6%, 71.9 ± 8.5% and 71.8 ± 9.7%, respectively (Figure 4.6b). Tianma concentration ≥ 62.5μg/mL could significantly reduce ($p < 0.001$) the DCFDA signal when compared with that of control.

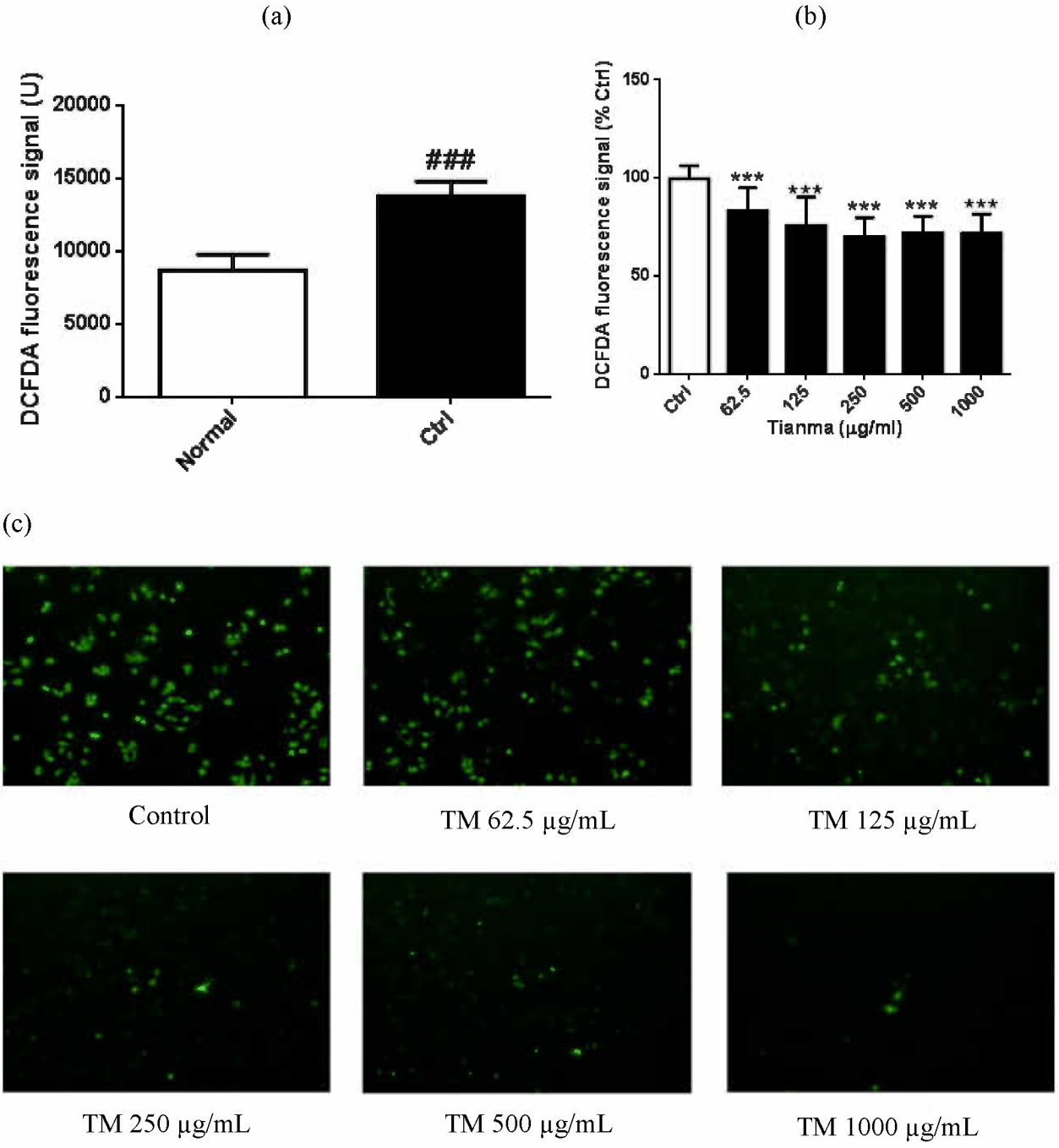


Figure 4.6 ROS production after OGD-induced oxidative stress on PC 12 cells. DCFDA signal of (a) OGD-induced oxidative stress between normal and OGD-treated group, (b) after OGD and Tianma-treatment on PC 12 cells. (c) Fluoresce images after incubation with DCFDA. Data were expressed as mean ± SD, n = 18 in 3 individual experiments. $^{###}p < 0.001$ as compared with control group by Student's t-test. ***$p < 0.001$ as compared with control by one-way ANOVA.

4.3.2 Anti-inflammation

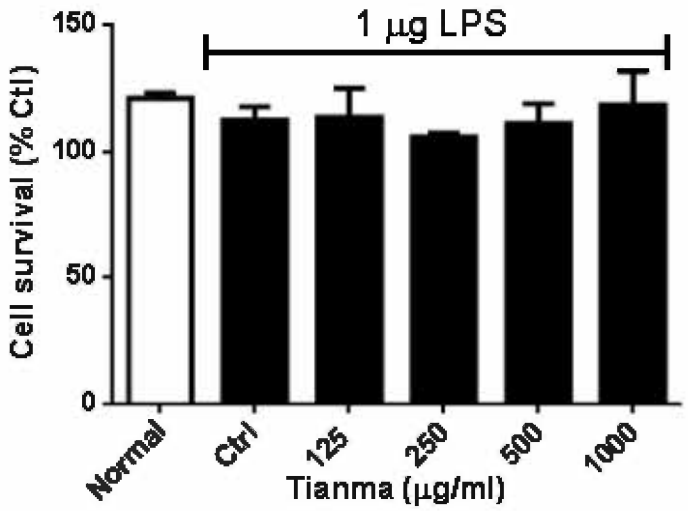
4.3.2.1 Measurement of nitric oxide (NO) production

The anti-inflammatory effects of Tianma extract after LPS-induced inflammation in RAW 264.7 cells were assessed by the NO production using Griess reagent. The results were shown in Figure 4.7.

The cytotoxicity of Tianma extract was assessed by the MTT assay. The cell survival of normal, control, Tianma treatment of 125, 250, 500 and 1000 μg/mL were 121 ± 2.2%, 112 ± 5.4%, 113 ± 11.6%, 106 ± 1.52%, 111 ± 8.2% and 118 ± 13%, respectively. No trend or significant changes of the cell survival after Tianma treatment was observed.

The percentage of relative NO production after LPS induction, compared with normal in RAW 264.7 cells in control, Tianma treatment of 125, 250, 500 and 1000 μg/mL were 100 ± 10.6%, 96.5 ± 10.7%, 87.8 ± 18%, 69.8 ± 15.5 and 61.2 ± 22.3%, respectively. A dose-dependent decrease in NO production was observed. The relative amount of stimulated NO production after 500 and 1000 mg/mL Tianma treatment were significantly ($p < 0.001$) reduced when compared with control.

(a)



(b)

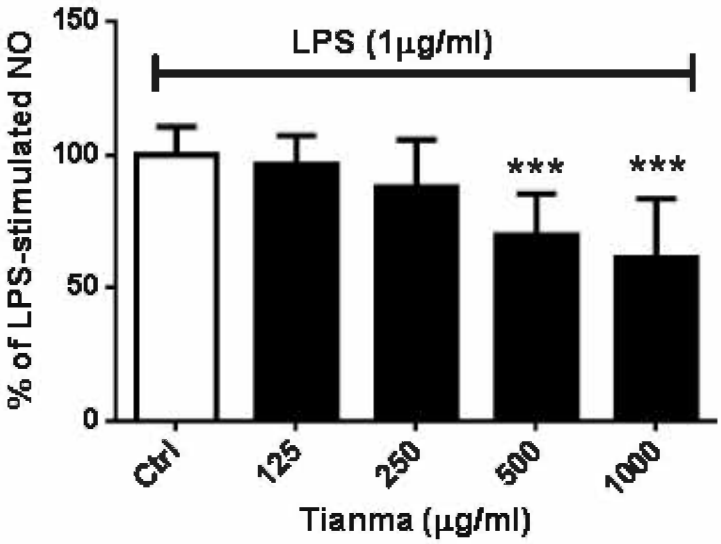


Figure 4.7 (a) The cytotoxicity of Tianma extract on RAW 264.7 cells (b) The effect of Tianma extract on LPS-induced NO production in RAW 264.7 cells. Data were expressed as mean ± SD, n = 18 in 3 individual experiments. ***$p < 0.001$ as compared with control by one-way ANOVA.

4.3.2.2 Measurement of pro-inflammatory cytokines by ELISA

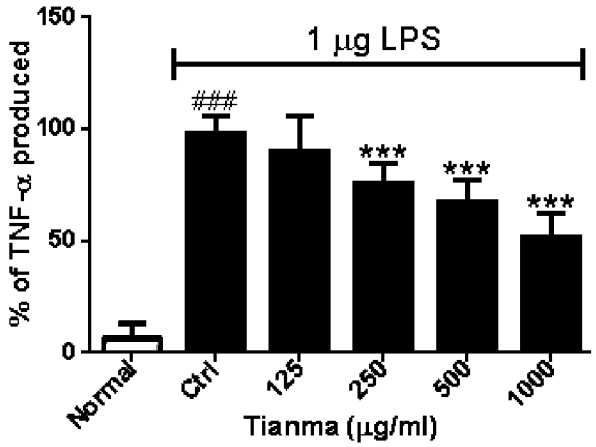
For measuring anti-inflammatory effect of Tianma in *in vitro* studies, pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in cell free supernatant were investigated by ELISA.

The concentration of TNF-α in the cell free supernatent from normal, control, Tianma treatment of 125, 250, 500 and 1000 μg/mL were 6.4 ± 6.8%, 98 ± 7.7%, 90 ± 16%, 75.8 ± 9%, 67.4 ± 10.1% and 51.7 ± 10.5%, respectively. The results on TNF-α production were shown in Figure 4.8a. TNF-α concentration of the control group significantly increased ($p < 0.001$) as compared with the normal group. A dose-dependent decrease of TNF-α concentration was observed after Tianma treatment. The Tianma extract of 250, 500 and 1000 μg/mL could significantly reduce ($p < 0.001$) the TNF-α concentration as compared with the control.

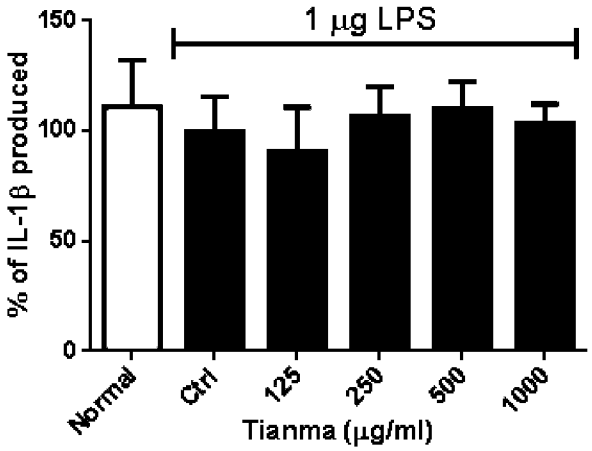
The concentration of IL-1β in the cell free supernatent from normal, control, Tianma treatment of 125, 250, 500 and 1000 μg/mL were 111 ± 21%, 100 ± 15.5%, 91 ± 19.7%, 107 ± 9.0%, 110 ± 10.1% and 103 ± 10.5%, respectively. The results were shown in Figure 4.8b. No significance increase of IL-1β production in control or dose-dependent effect was observed.

The results on IL-6 production were shown in Figure 4.8c. The concentration of IL-6 in the cell free supernatent from normal, control, Tianma treatment of 125, 250, 500 and 1000 μg/mL were 2.77 ± 12.1%, 100 ± 5.37%, 90.1 ± 11.1%, 88.7 ± 8.98%, 73.9 ± 18.9% and 64.1 ± 21.8%, respectively. IL-6 concentration of the control group significantly increased ($p < 0.001$) as compared with the normal group. A dose-dependent decrease of IL-6 concentration was observed after Tianma treatment. The Tianma extract of 500 and 1000 μg/mL could significantly reduce ($p < 0.001$) the IL-6 concentration as compared with the control.

(a)



(b)



(c)

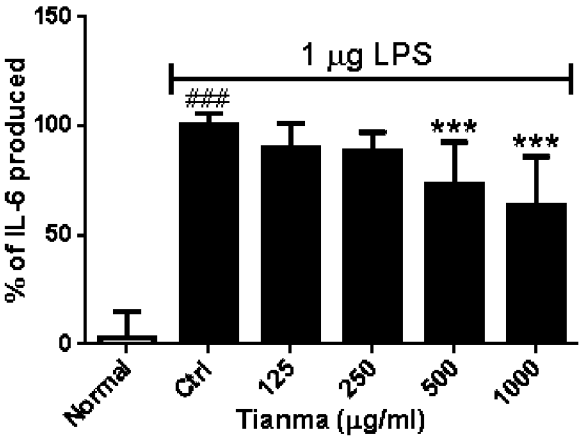


Figure 4.8 The effect of Tianma treatment on the pro-inflammatory cytokines levels in RAW 264.7 cells after LPS-induced inflammation. (a) TNF-α level, (b) IL-1β level and (c) IL-6 level. Data were expressed as mean ± SD, n = 18 in 3 individual experiments. $^{###}p < 0.001$ as compared with normal group by Student's t-test. ***$p < 0.001$ as compared with control by one-way ANOVA.

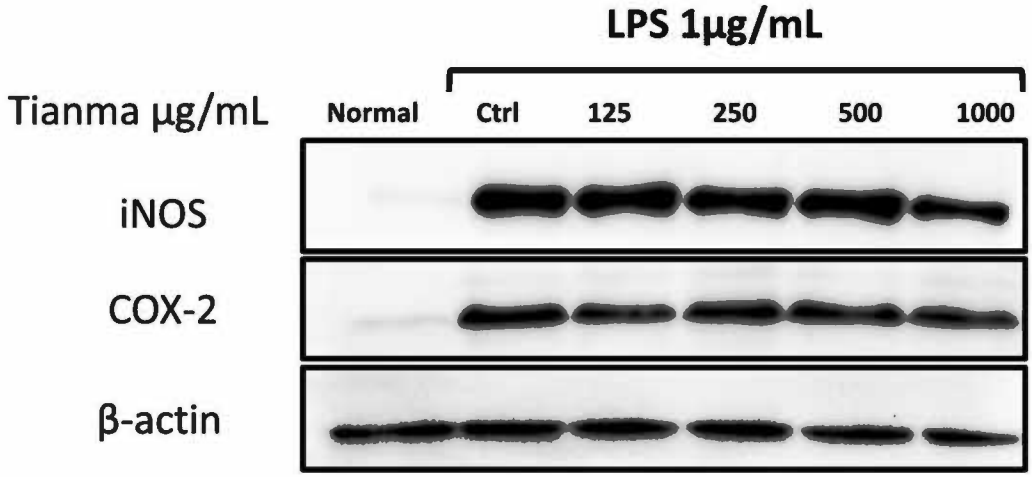
4.3.2.3 Determination of inflammatory factors inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression

In vitro studies on the effect of Tianma extract on inflammatory factors iNOS and COX-2 expression after LPS-induced inflammation on RAW 264.7 cells were investigated by Western blotting (Figure 4.9a).

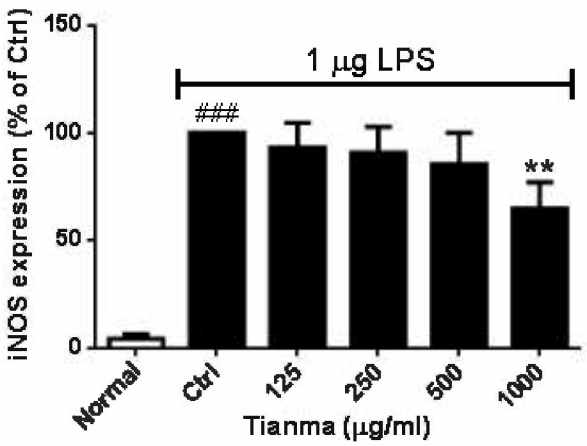
The expression of iNOS in whole cell protein from normal, control, Tianma treatment of 125, 250, 500 and 1000 μg/mL, normalized with β-actin were 4.27 ± 2.1%, 100 ± 0%, 93.3 ± 11.3%, 90.8 ± 12.1%, 85.4 ± 14.5% and 64.7 ± 12.4%, respectively. The results were shown in Figure 4.9b. iNOS expression of the control group significantly increased ($p < 0.001$) as compared with the normal group. A dose-dependent decrease of iNOS expression was observed after Tianma treatment. The Tianma extract of 1000 mg/mL could significantly reduce ($p < 0.01$) the iNOS expression induced by LPS.

The expression of COX-2 in whole cell extract from normal, control, Tianma treatment of 125, 250, 500 and 1000 μg/mL, normalized with β-actin were 8.6 ± 3.2%, 100 ± 0%, 88.8 ± 9.1%, 89.4 ± 9.7%, 79.5 ± 8.0% and 72.9 ± 8.4%, respectively. The results were shown in Figure 4.9c. COX-2 expression of the control group significantly increased ($p < 0.001$) as compared with the normal group. A dose-dependent decrease of COX-2 expression was observed after Tianma treatment. Tianma extract of 500 and 1000 μg/mL could significantly reduce ($p < 0.01$ and $p < 0.001$ respectively) the COX-2 expression induced by LPS.

(a)



(b)



(c)

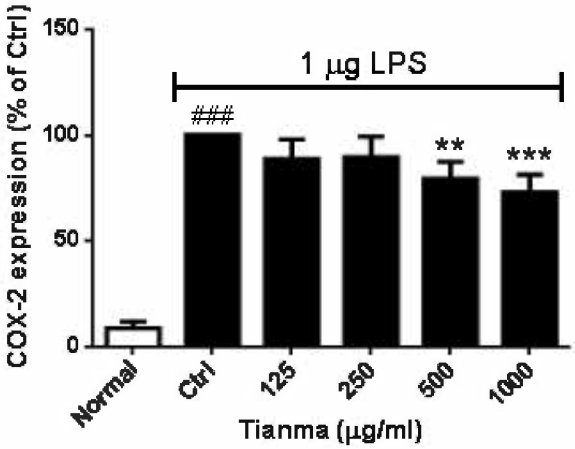


Figure 4.9 The effect of Tianma extract on LPS-induced iNOS and COX-2 expression in RAW 264.7 cells. (a) Representative blots showed the iNOS and COX-2 expression (b) Relative iNOS expression (c) Relative COX-2 expression. Data are expressed as mean ± SD, n = 4 in 4 individual experiments.. $^{###}p < 0.001$ as compared with normal group by Student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with control by one-way ANOVA.

4.4 Discussion

In this chapter, we performed several experiments to investigate the cellular mechanistic pathway on anti-oxidation and anti-inflammation in response to the promising neuroprotective effect of Tianma water extract we observed in the MCAo rat model.

PC 12 cells were selected for H_2O_2-induced oxidative stress model and OGD model. PC 12 cells were extensively used in neuroprotection studies regarding cerebral ischemia and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Refolo *et al.*, 1989; Walkinshaw and Waters, 1995). PC 12 cells were also widely used for cell signaling studies (Vaudry *et al.*, 2002). Moreover, it has been reported that PC 12 cells were suitable to be used in the OGD model as they were sensitive to the hypoxia and provide corresponding response (Seta *et al.*, 2002)

Hydrogen peroxide (H_2O_2) is produced by mitochondria (Boveris and Chance, 1973) under normal situations. The produced H_2O_2 would be scavenged by anti-oxidative enzymes such as SOD, GPx and catalase. Resupply of oxygen occurred during reperfusion provides a substrate for various enzymatic oxidation reactions leading to accumulation of excess reactive oxygen radicals including H_2O_2. Many reactive oxygen radicals participated in neuronal cell injuries in cerebral ischemia and reperfusion (Chan, 1994; Chan, 2001), resulting in neuronal cell damage and death in cerebral ischemia and reperfusion (Saito *et al.*, 2005). Besides, the breaking down of accumulated superoxide in ischemic brain tissue by SOD would also lead to the formation of H_2O_2 (Fridovich, 1995). As H_2O_2 has a high mobility and could go across cell membranes freely, it could undergo the Haber–Weiss reaction and be converted into other free radicals such as hydroxyl radical ($OH^{\cdot}$) (Halliwell, 1992). The presence of H_2O_2 has been proven to be closely related to the infarct size and apoptosis (Crack *et al.*, 2001). How to reduce accumulated

H_2O_2 after ischemia-reperfusion could be a potential therapeutic pathway for stroke. For example, the presence of GPx and CAT contributes to the decrease of accumulated H_2O_2 as it can be metabolized into water or water and oxygen by GPx and CAT respectively (Brannan *et al.*, 1981; Marchena *et al.*, 1974). H_2O_2-induced oxidative stress model was an extensively used (Kil *et al.*, 1996; Schinzel *et al.*, 2005) to mimic the oxidative stress during reperfusion and to investigate the effect of drugs under this condition (Vannucci, 1990).

From MTT assay, studying of cell viability on PC 12 cells after treatment with different H_2O_2 concentration (Figure 4.4a), H_2O_2 induced cytotoxicity on PC 12 cells was significant. This finding was consistent with similar studies (Hong and Liu, 2004; Yu *et al.*, 2008b) and could be explained by high level of oxidative stress exerted by the elevated H_2O_2, which was beyond the protective capacity of anti-oxidation, followed by induced apoptotic cellular pathway due to mitochondrial dysfunction, finally leading to neuronal cell death (Maroto and Perez-Polo, 1997; Tong and Perez-Polo, 1996). From MTT assay studying PC 12 cell viability after treated with Tianma extract (Figure 4.4b), the result showed that a high level of Tianma extract (1000 μg/mL) caused no toxicity to the PC 12 cells. Moreover, the cell viability after being treated with Tianma extract (125 to 1000 μg/mL) was even higher than the control group. We suggested that the Tianma water extract could increase the PC 12 cells proliferation under normal incubation condition. From a previous study, the active ingredient from Tianma, Gastrodin, showed no toxic effect to neuronal cells and was neuroprotective (Zhao *et al.*, 2012). After H_2O_2-induced oxidative stress in PC 12, the Tianma treatment could significantly increase the cell survival (Figure 4.4c). This implied that Tianma water extract had anti-oxidative effects, probably by acting as an ROS scavenger. It may be related to the stimulation of Tianma water extract on GPx and CAT enzymatic activities as what we observed

in the MCAo model in previous chapter (Figure 3.13 and Figure 3.14).

Glucose and oxygen are essential nutrients for neuronal cell survival and normal function. Oxygen-glucose deprivation (OGD) model is one of the most widely used *in vitro* cell model (Bruer *et al.*, 1997; Grabb and Choi, 1999; Zhou *et al.*, 2008) for mimicking a stroke attack. This is considered as the evaluation of cerebral ischemia and its consequences from a cellular mechanistic aspect (Graham *et al.*, 2004). The OGD model we used in this study was based on the one proposed by Hillion *et al.* (Hillion *et al.*, 2005). The duration design experiment (Figure 4.5a) allowed us to find the optimal condition, maximizing the cell death to 90%. This "standard" was widely accepted as the basal cell survival for drug-screening in OGD model (Abu-Raya *et al.*, 1999; Tabakman *et al.*, 2002; Tabakman *et al.*, 2005). Although the ischemic duration used in the *in vivo* MCAo study was 2 hours, various studies reported that 2 hours ischemia would not be enough for *in vitro* OGD model to achieve a significant cell death for anti-ocidative study (Abu-Raya *et al.*, 1999; Hillion *et al.*, 2005; Tabakman *et al.*, 2002). From the cell survival after Tianma extract with at the presence or absence of 16 hours ischemia and followed by 24 hours reperfusion on PC 12 cells (Figure 4.5b), the Tianma extract could significantly rescue cells from death induced by ischemia reperfusion process.

2',7'-dichloro-dihydrofluorescein diacetate (H_2-DCFDA or simply called DCFDA) assay could be used to measure the ROS amount, and was considered as an indirect way to reflect the ROS scavenging property. DCFDA hasn't been reported that it would have any interaction with Tianma chemical compounds, but it has been reported that it would interact with organic solvents such as ethanol and DMSO (Brömme *et al.*, 2008). When nonpolar DCFDA was applied to intact cells, it would cross the cell membrane and hydrolyzed by intracellular esterases to nonfluorescent DCFH. With the presence of ROS, the DCFH would be further oxidized into highly

fluorescent dichlorofluorescein (DCF). The ROS amount thus could be quantified by the fluorescent signal (Bass *et al.*, 1983; LeBel *et al.*, 1992). From the result of ROS production in PC 12 cells after OGD (Figure 4.6a), OGD would induce the production of ROS in PC 12 cells significantly which was consistent with the establishment of OGD model in other study (Liu *et al.*, 2003). In Figure 4.6b, we noted that the Tianma extract could significantly reduce the ROS amount. We suggested that the Tianma extract could increase the ROS scavenging property in PC 12 cells or even serving as free radical scavenger or anti-oxidant. This meant that Tianma water extract had similar effect as ethanolic extract and diethyl ether fractions of Tianma, which had been proven to function as an anti-oxidant and free radical scavenger for OH · , respectively (Kim *et al.*, 2003; Liu and Mori, 1992). Similar study had been performed on the Gastrodin (GAS) on OGD-treated cells. The cell lines used and the assessment assay however were different from our set-up. This made it hard to be compared between these studies (Zeng *et al.*, 2006). As OGD model induced apoptosis in PC 12 (Hillion *et al.*, 2005), we believed that the Tianma water extract had the anti-apoptotic properties. Therefore, we tried to explore if there is any anti-apoptotic property in Tianma extract. The result was reported the next chapter, chapter 5.

Inflammation of the brain primarily involved the participation of the two types of glial cells, microglia and astrocytes. Under normal conditions, microglia acts as surveillance for immune response in CNS, while astrocytes serve a role of maintaining ionic homeostasis, buffering the action of neurotransmitters and release of nerve growth factors (Aloisi, 1999; Kreutzberg, 1996). When ischemia occurred, series of inflammatory response, including the activation and proliferation of these glia cells and the process was known as gliosis (Perry and Gordon, 1991), acting as a defense mechanism to restore blood supply (Norton *et al.*, 1992; O'Callaghan, 1991).

However, the activated glia cells were responsible for the production various produce pro-inflammatory and neurotoxic factors, including cytokines, such as TNF-α, IL-1β and IL-6 and free radicals, such as NO and superoxide (Banati *et al.*, 1993; Minghetti and Levi, 1998).

RAW 264.7 cells were selected for lipopolysaccharide (LPS)-induced inflammation mechanistic study. RAW 264.7 cells were extensively used as a platform for various inflammation studies including NO production, NF-κB modulation and inflammatory cytokines production (Huo *et al.*, 2012; Wang and Mazza, 2002; Yang *et al.*, 2010).

LPS, known as prototypical endotoxin, localized at the outer membrane of Gram-negative bacteria, such as *E. coli.*. LPS could induce a strong immune response in normal mammalian cells (Galanos *et al.*, 1985) by direct activation of macrophages (Corriveau and Danner, 1993). The LPS-induced inflammation was extensively applied in neuroscience research (Franklin *et al.*, 2003; Liu *et al.*, 2002a; Villa *et al.*, 2003). It was proven that the inflammation induced by LPS would lead to the production of inflammatory cytokines, such as IL-6 and TNF-α (Lawrence *et al.*, 2002), as well as effectors such as COX-2 and iNOS (Ahn and Aggarwal, 2005; Gao *et al.*, 2012). Inflammatory pathway could be initated by pro-inflammatory cytokines such as TNF-α, IL-1β, etc. (Wood and Rothwell, 1998; Zoppo *et al.*, 2000b) as well. It has been reported LPS and pro-inflammatory cytokines had similar effects in upregulating inflammatory protein and gene expression on *in vitro* studies (Matsuguchi *et al.*, 2000; Sheng *et al.*, 2011) but manipulation of LPS was at a lower cost. We used LPS to induce inflammation in RAW 264.7 cells and observed whether the Tianma extract could exhibit any anti-inflammatory effects via various mechanisms with this platform. From the result (Figure 4.7a), no cytotoxicity from Tianma extract was observed.

NO production is mediated by nitric oxide synthase (NOS), catalyzing the conversion of arginine with molecular oxygen to give rise to citrulline and NO (Bredt, 1999). NO serves various roles in physiological functions, such as immune modulations, muscle relaxation as well as neuronal activity (Grisham *et al.*, 1999). However, NO is a free radical. When ischemia occurred, the activated glial cells and the high intracellular Ca^{2+} stimulated NOS (Choi, 1995) would upregulate the NO production. NO would react with superoxide to give rise to a highly reactive free radical peroxynitrie anion (ONOO-) (Ischiropoulos, Zhu, & Beckman, 1992).

In our study, we investigated whether Tianma water extract could reduce NO production after LPS-induced inflammation for screening its anti-inflammatory effect. The result (Figure 4.7b) showed that the Tianma extract could significantly reduce NO production dose-dependently. The finding was similar to the effect of ethanolic extract of Tianma (Ahn *et al.*, 2007). This provided us with a preliminary evidence for further investigation on the Tianma.

The release of cytokines would further exacerbate the inflammation. This was due to interaction between cytokines and activated glial cells were close to each other, as the glial cells were responsible for the production of cytokines which could activate the glial cells in return (Eng, 1988; Sawada *et al.*, 1989). More neutrophils would migrate to the infarct area and further intensify the inflammation (Suk, 2007). The gliosis could be induced by pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 (Balasingam *et al.*, 1994). The expressions of these pro-inflammatory cytokines were associated with the degree of brain injury (Feuerstein *et al.*, 1998). Therefore, we used ELISA to measure the effects of Tianma extract on the pduction of TNF-α, IL-1β and IL-6. The results (Figure 4.8) showed that Tianma extract could significantly reduce the pro-inflammatory cytokines TNF-α and IL-6 concentration in a dose-dependent manner after LPS-induced inflammation. It has been reported that

Gastrodin had similar effect on TNF-α expression but on different cell lines and assays (Dai *et al.*, 2011). We suggested that Tianma water extract attenuate the cytokines level by inhibiting NF-κB signaling pathway and phosphorylation of MAPKs, as GAS had been proven to be anti-inflammatory via these pathways (Dai *et al.*, 2011; Yang *et al.*, 2013). However, this hypothesis required further investigation. There was no elevation of IL-1β level after LPS-induction. According to the manufacturer, analytical sensitivities for TNF-α, IL-6 and IL-1β were all ≥ 10 ng/mL. However, the actual performance of the IL-1β assay in this study had not reached the proposed level for our samples. We suggested these might be due to the variation of set performance substantially from that cited by the manufacturer with low sensitivity of the ELISA kit or only trace amount of IL-1β presented in the samples.

As mentioned, the synthesis of NO is controlled by NOS, including iNOS, eNOS and nNOS (Bredt, 1999). Among these three NOS, we are interested in the regulation of iNOS by Tianma extract as iNOS is highly expressed in glial cells and macrophages (Yoon *et al.*, 2009). Besides NO, during inflammation, another inflammatory effector, cyclooxygenase-2 (COX-2) is also upregulated which is closely related to PGE_2, an inflammatory mediator. The COX-2 expression is also upregulated by the glial cell during inflammation (Murakami and Ohigashi, 2007). Therefore, we investigated if there was inhibitory effect of Tianma water extract on the iNOS and COX-2 expression by Western blotting. As the result shown (Figure 4.9), the Tianma water extract could significantly reduce the expression of these two inflammatory effectors dose-dependently. The result was similar to other related studies on Tianma ethanolic extract (Ahn *et al.*, 2007).

In conclusion, Tianma extract used in our study showed an anti-oxidative effect through ROS scavenging and an anti-inflammatory effect, by downregulating pro-inflammatory cytokines TNF-α and IL-6, inflammatory effectors iNOS and

COX-2. The neuroprotective effects of Tianma extract on MCAo rat model might be related to both of these two factors. However, further studies are required to confirm the precise mechanisms of Tianma extract for ischemic stroke.

Chapter 5 General discussion

5.1 General discussion and conclusion

Stroke is a non-communicable disease as well as one of the increasing health burden-causing disease of the world. According to World Health Organization (WHO), stroke accounts for about 9.7% of the world's total death in 2004 and was also known as the second leading cause of death over the world (Mathers *et al.*, 2008). From estimation, the number of global deaths has been projected accordingly to rise to 6.5 million in 2015 and to 7.8 million in 2030 (Strong *et al.*, 2007).

There are numerous cellular injury mechanisms accompanied with the cerebral ischemia and reperfusion, including oxidation, inflammation, apoptosis, etc. They are closely interacted with each other. The western conventional treatment of ischemia is focusing on perfusion and reduction of neuronal cell death (Ferriero, 2004; Moskowitz *et al.*, 2010). However, patients might be exposed to a higher risk of having intracerebral hemorrhage after rt-Pa treatment (NINDS rt-PA Stroke Study Group, 1995). No one-step stroke treatment drugs targeting on anti-oxidation, anti-inflammation and anti-apoptosis pathways had gained USFDA approval for acute ischemic stroke treatment yet (Roach *et al.*, 2010). Multi-targeting drugs are required to solve this dilemma. There were some Chinese medicines long been in use for stroke prevention and treatment (Zhou and Xiao, 1997) and had been proven to have neuroprotective effects (Huang *et al.*, 1994a), with few or no side-effects (Xu, 1991). In TCM clinical practice, Tianma was usually used to cure neurasthenia, headache, paralysis, hemiplegia, epilepsy, etc. (Wu *et al.*, 2007). Tianma crude organic extracts and components from organic solvent isolation showed positive neuroprotective effects towards ischemia via different mechanisms (Huang *et al.*, 2007; Kam *et al.*, 2011; Kim *et al.*, 2007; Tsai *et al.*, 2011; Yu *et al.*, 2010), but no related studies on the effects of Tianma water extract have been performed. In this

study, we tried to investigate the *in vivo* and *in vitro* neuroprotective effects of Tianma water extract.

The first part of our study employed the middle cerebral artery occlusion (MCAo) to mimic the transient cerebral ischemia by insertion of an intraluminal filament (suture) to the origin of the MCA without craniectomy (Longa *et al.*, 1989; Schmid-Elsaesser *et al.*, 1998). In our experimental procedure, rats were pretreated with Tianma water extract one hour before occlusion of the middle cerebral artery to induce 2 hours of ischemia and followed by 24 hours of reperfusion. This model would induce neuronal damage by oxidation and inflammation (Vakili *et al.*, 2011; Yamato *et al.*, 2011). Therefore, the brain infarction and neurological score would be investigated as well as the determination of anti-oxidative effect of Tianma water extract via the study on anti-oxidative enzymes SOD, GPx and CAT as well as the anti-inflammatory effect via studying of pro-inflammatory cytokines TNF-α and IL-6. We found that Tianma water extract could significantly ameliorate the brain infarct as well as neurological deficits probably by significantly attenuating the production of pro-inflammatory cytokines TNF-α and IL-6 and enhancing the release of anti-oxidative enzymes SOD, GPx and CAT.

Due to the prominent neuroprotective effects of Tianma water extract on regulating anti-oxidative enzymes as well as pro-inflammatory cytokines in the MCAo model, we used *in vitro* studies to verify the cellular mechanisms underlying these observations by studying the oxidative stress due to production of free radicals and inflammation closely related to the rise of free radicals. We performed H_2O_2-induced oxidative stress model and oxygen-glucose deprivation (OGD) model on PC 12 cells. The former model could mimic the production of ROS during ischemia, while the latter model could mimic the ischemic condition as well as effects on the reperfusion. We found that Tianma water extract could significantly

increase cell viability under the ischemic condition and enhanced the ROS scavenging ability. For inflammation mechanistic study, Lipopolysaccharide (LPS)-induced inflammation was performed on marcophage RAW 264.7 cells. The Tianma water extract could significantly reduce the NO production and downregulate pro-inflammatory cytokines TNF-α and IL-6 as well as inflammatory effectors iNOS and COX-2.

Once ischemic stroke had begun, reoxygenation to the infarct area would cause ROS formation which led to oxidative damage. This would result in blood-brain barrier dysfunction and microvascular injury, which in turn led to the post-ischemic inflammation (Dirnagl *et al.*, 1999) and even worsen the neuronal damage (Del Zoppo *et al.*, 1991). Our findings showed the anti-oxidative and anti-inflammatory effects of Tianma water extract, suggesting that Tianma water extract could ameliorate the ROS production or enhance the ROS scavenging properties and suppressed post-ischemia inflammatory response including upregulation of NO and inflammatory cytokines, could be suppressed (Dirnagl *et al.*, 1999; Waxman, 1996). This enabled Tianma water extract to provide early neuroprotective effect before the neuro-inflammation occurred. Moreover, the downregulation of iNOS and COX-2 by Tianma water extract could directly reduce the formation of the destructive NO and inflammation-related superoxide, respectively (Iadecola, 1997; Nogawa *et al.*, 1997), thus reducing the post-ischemic inflammatory damages (Dirnagl *et al.*, 1999).

In conclusion, our study provided the first *in vivo* and *in vitro* scientific evidence showing the potent neuroprotective effects of Tianma water extract towards ischemic stroke via anti-oxidation and anti-inflammation mechanisms. This result would be a clinical benefit to ischemic stroke patients as Tianma might be a potential multi-targeting drug towards this disease. However, further studies were required to elucidate the upstream regulation of cellular pathological mechanisms.

5.2 Significance of the study

Majority of previous studies were focused on Tianma organic crude extracts or its components. However, traditional way of taking Chinese Medicine was using herbal water extract, which might be more suitable than organic extracts for human intake. Moreover, the chemical components from the water extract may differ from those of organic extract or there were even conformational change during extraction. Our group has compared the chemical profiles of different extraction method, showing that Tianma water extract could extract the highest amount of Gastrodin (2.87% v/v) among ethanol (1.40% v/v) and methanol extract (0.024%) (Huang *et al.*, 2007; Wu *et al.*, 2007).

From various studies, Gastrodin have been proven to be the active compotent for anti-stroke effects by different mechanistic studies, such as downregulating caspase-3 expression, inhibition on MAPK pathways, protection on hypoxia-induced toxicity, etc. (Bin, 2011; Dai *et al.*, 2011; Xu *et al.*, 2007). However, when we compared the neuroprotective effects in MCAo rat model, we found that Tianma water extract achived a better effect than Gastrodin alone on the improvement of neurological score and reduction of brain infarct volume (Zeng *et al.*, 2006).

Furthermore, Tianma water extract may have positive influence on blood circulation. The risk of ischemic stroke was highly associated with different circulatory diseases, such as hypertension (Sacco, 1997; Sacco *et al.*, 1997), coronary artery disease and cardiac hypertrophy (Pocock *et al.*, 1988; Soler and Ruiz, 2010), etc. We suggested that Tianma water extract could contribute to the amelioration of circulation. Tianma extract and Gastrodin has been reported to modulate blood vessel tonicity by stabilizing the arterial structure and enhancing blood vessel elasticity (Feng *et al.*, 2012), protects against cardiac hypertrophy (Shu *et al.*, 2012), inhibits vascular smooth muscle cell proliferation (Zhu *et al.*, 2012) and asserts anticoagulant

effects (Liu *et al.*, 2006).

We suggested that no single compound could tackle all anti-stroke mechanisms, but the application of Tianma water extract might be a new direction for ischemic stroke therapy. As there might be other unidentified chemical compounds present in Tianma water extract and the synergistic effects among various compounds in Tianma water extract, such as GAS and 4-Hydroxybenzaldehyde (4-HBAL), etc. could continue as therapeutic agents towards ischemic stroke.

This investigation on the Tianma water extract is novel towards ischemic stroke. We were the first group to demonstrate the *in vivo* neuroprotective effects of Tianma water extract in MCAo model in which amelioration of the neurological defect was observed. We also demonstrated for the first time the anti-oxidative and anti-inflammatory effects of Tianma water extract in this model. In addition, this is the first report of the *in vitro* anti-oxidative and anti-inflammatory effects of Tianma extract using oxygen-glucose deprivation model and LPS-induced inflammation model. These observations allowed us to explore the therapeutic effect of Tianma water extract as an entity for stroke patients. It also opened up the potential of using Tianma water extract as a multi-target therapeutic agent for ischemic stroke and established Tianma water extract for stroke patients and to prevent recurrence of second stroke.

5.3 Limitations of the study and future work

In our study, normal young rats were used. On the other hand, ischemic stroke is believed to be related to age and chronic diseases such as hypertension. In order to study whether the Tianma extract would interfere with the effect of age and hypertension when compared to our study, elder spontaneously hypertensive rats (SHRs) should be used to provide a reference which is more close to the reality.

The removal of filament in MCAo model to achieve reperfusion undoubtedly differed from the clinical condition as the reperfusion is resulted from thrombolysis (Warach and Latour, 2004). The more suitable approach to address the reperfusion due to thrombolysis could be achieved by the insertion of externally formed blood clot to the MCA by a catheter (Wang-Fischer, 2008). However, it is difficult for the researchers to locate the precise location of the ischemia as the size of blood blot is hard to control. The complexity of this model is much higher than that of MCAo.

Some ELISA kits used in this study showed low sensitivity or even undetectable signals. This may be due to the low sensitivity of the kits or only trace amount of target cytokines present in the sample. Western blot or real time PCR could be used to study the expression of the protein or the RNA.

One of the treatments on ischemic stroke is the thrombolysis. We did not perform any investigation of the thrombolytic effect of Tianma water extract in our study. However, we suggested that the reduction of brain infarct may be due to the anti-thrombotic effect of Tianma extract as some previous studies showed that some isolated components from Tianma or Tianma herbal formula had this effect (Ding *et al.*, 2007; Yang *et al.*, 2009). The anti-thrombotic effect of Tianma water extract should be investigated in the future.

As Tianma water extract could lower the release of NO and pro-inflammatory cytokines in our studies, we suggested that this regulation may be via APK Pathways,

so as the downregulation of downstream transcription factors, nuclear factor-κB (NF-κB) and cyclic AMP-responsive element (CRE)-binding protein (CREB) by blocking the phosphorylation (Dai *et al.*, 2011). Verification should be done in the future study.

Besides the typical apoptotic pathways of tissue damage, ROS and NO were proven in previous studies that they were acting as mediators to regulate the necrotic and apoptotic cell death after ischemic stroke (Buttke and Sandstrom, 1994; Chopp *et al.*, 1996; Stamler *et al.*, 1992). Therefore, we also performed some investigations on the anti-apoptotic effects of Tianma water extract by using OGD model. The details of this study would be described in the supplementary chapter.

Last but not least, necrosis was known to play one of the most important roles in ischemic cell death (Hou and MacManus, 2002; Lassen, 1982). Necrotic cell death was considered as a passive cellular degeneration due to internal homeostasis collapses (Hou and MacManus, 2002), such as irreversible cell swelling, endoplasmic reticulum dilation, increased mitochondrial density, etc. which would in turn lead to the rupture of nuclear membranes (Aggarwal *et al.*, 2010; Choi *et al.*, 2009; Hou and MacManus, 2002) as well as plasma membrane. Eventually the cell debris would be ingested and degraded by phagocytes (Aggarwal *et al.*, 2010; Ting *et al.*, 1983; Zamzami *et al.*, 1997). On the other hand, necrosis would cause the release of intracellular enzymes into the liberation of cellular enzymes into the interstitium and lead to inflammatory response (Zamzami *et al.*, 1997). Moreover, the cells surrounding the necrotic core will undergo apoptotic cell death within a short period of time (Broughton *et al.*, 2009). Therefore, further investigations should be performed by flow cytometry (annexin V-FITC/propidium iodide) to distinguish the apoptotic and necrotic cell death after OGD-Tianma treatment (Chan *et al.*, 2011), as well as further identify primary and secondary necrotic cells by analysis of release of

cytokeratin 18, HMGB1, etc. (Krysko *et al.*, 2008). This will enable us to explore more evidence for using Tianma water extract as therapeutics for ischemic stroke via anti-necrotic pathway.

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Supplementary chapter *In vitro* study on anti-apoptotic effects of Tianma extract on ischemic stroke

S.1 Introduction

Apoptosis is known as "the programmed cell death" which undoubtedly played an important role in the cellular injury of ischemic stroke. During ischemic stroke incident, the brain tissue exposed to the greatest reduction of blood flow and neurons underwent necrotic cell death, known as "necrosis". The less severely affected tissue surrounding the core still remained metabolically active (Ginsberg, 1997), which was known as "ischemic penumbra". Ischemic penumbra surrounding the necrotic core would undergo apoptotic cell death within a short period of time. Apoptosis was known to be initiated in ischemia-reperfusion stage (Broughton *et al.*, 2009). Similar findings in previous studies of MCAo demonstrated that apoptosis was associated with the cerebral ischemia (Xu *et al.*, 2005; Yin *et al.*, 2003). There were two general pathways for mediation of apoptosis, the intrinsic and extrinsic pathways (Broughton *et al.*, 2009). A potential post-stroke treatment to reduce neuronal cell death was by preventing neuronal cells entering apoptotic phase or terminating the apoptotic pathway, including the intrinsic and extrinsic pathways (Broughton *et al.*, 2009; Ginsberg, 1997).

Apart from inducing oxidative stress and damaging neuronal cells (See Chapter 4), ROS were also related to ischemia-induced apoptosis (Mergenthaler *et al.*, 2004). The pro-inflammatory cytokines, such as IL-1β, TNF-α had been proven to play a vital role in the apoptotic pathway (Broughton *et al.*, 2009; Doyle *et al.*, 2008) and triggered apoptotic neuronal cell death (Licinio, 1997). Therefore, apoptosis is not just an independent event in ischemic stroke, but a consequence of cell injuries as well.

On the other hand, OGD-reperfusion model on PC 12 could also activate the

apoptotic pathway (Hillion *et al.*, 2005). We tried to further investigate if there was any anti-apoptotic effect from Tianma water extract. Some previous studies on chemical components of Tianma or its ethanolic extract observed anti-apoptotic effects *in vivo* or *in vitro* (Tsai *et al.*, 2011; Yu *et al.*, 2010), but no studies on Tianma water extract have yet been done.

In this chapter, we investigated the anti-apoptotic effects of Tianma water extract by using flow cytometry as well as the expression of two apoptotic proteins, anti-apoptotic Bcl-2 and pro-apoptotic Bax after OGD treatment.

S.2 Materials and methods

S.2.1 Rat pheochromocytoma PC 12 cells

Rat pheochromocytoma PC 12 cells were purchased from ATCC and were maintained at subconfluent density in RPMI-1640. The details of culture method were the same as those stated in section 4.2.2.1.

S.2.2 Measurement of cell proliferation and apoptosis by flow cytometry

PC 12 cells were seeded at $3x10^5$ cells/well in 6-well plates precoated with poly-L-lysine overnight in complete RPMI 1640 culture medium with 10% v/v horse serum, 5% v/v FBS and 1% v/v Pen Strep at 37°C with 5% CO_2 and 95% air. Then the cells were exposed to oxygen glucose deprivation (OGD) and reperfusion model as mentioned in section 4.2.2.3 for 16 hours ischemia and 24 hours reperfusion.

The cell proliferation and apoptosis were measured by the Apoptosis, DNA Damage and Cell Proliferation Kit purchased (BD Biosciences, San Diego, CA, USA). The kit was used according to manufacturer's instruction. Reagents used were supplied by the manufacturer except specified.The protocol was summarized as follow. After OGD, cells were washed with PBS, trypsinized and centrifuged at 1500

RPM for 3 mins. Cells were resuspended in RPMI 1640 culture medium and then fixed and permeabilized the cells by incubation with BD Cytofix/Cytoperm Fixation/Permeabilization Solution for 30 mins on ice. Cells were washed by adding BD Perm/Wash Buffer, followed by centrifuge at 250 x g for 5mins. Then supernatant were discarded. Cells were resuspended and incubated with BD Cytofix/Cytoperm Plus Permeabilization Buffer for 10 mins on ice. After washing with Perm/Wash Buffer, cells were then centrifuged at 250 x g for 5mins. The cells were resuspended and re-fixed again by Cytofix/Cytoperm Fixation/Permeabilization solution for 5 mins on ice. Cells were subsequently washed again and centrifuged down, with supernatant were discarded. 300 μg/mL DNase were incubated with cells for 1 hour at 37 ^{o}C. After further washing by Perm/Wash Buffer, cells were centrifuged at 250 x g for 5 mins. Resuspended cells were stained with rection mixture which included BD Perm/Wash Buffer, PerCP-Cy™5.5 Anti-BrdU and PE Anti-Cleaved PARP antibodies. Cells were washed after 20 mins of incubation at room temperature. Staining buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO4, 2mM KH_2PO4, 13.84mM NaN_3, 2% heat inactivated FBS) was added to samples.

Cells were analysed with a flow cytometer (BD FACSCanto™ II system Firmware Version 1.47, BD Biosciecnces, San Diego, CA, USA) and the manufacturer software (BD FACSDiva Software Version 6.1.2, BD Biosciecnces, San Diego, CA, USA). Data were collect from 10000 events. The apoptosis data was processed by WinMDI 2.9 (The Scripps Research Institute, California, USA). DNA results from flow cytometry for the cell proliferation were further analyzed by ModFit LT Version 3.0 (Verity Software House, Topsham, Maine, USA).

S.2.3 Determination of anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax

Whole cell protein extracts were prepared by lysing the cells with RIPA lysis buffer as described in Chapter 4.2.2.4. Protein concentration in the extracts was quantified by the BCA assay as described in section 3.2.5.2.

Equal amount of protein extracts, 40 μg of whole cell extract, were separated by a 10% SDS-Polyacrylamide gel electrophoresis. The gel was then transferred onto a PVDF membrane. The transblotted membranes were blocked with 0.5% non fat milk in TBST for 1 hour. The membranes were incubated overnight at 4 °C with specific antibodies against Bax, Bcl-2 and β-actin (Cell Signaling Technology, Danvers, MA, USA). The blots were then washed three times with TBST before incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) for 2 hours. The blots were then washed another three times with TBST before developing. The blots were developed by using Amersham ECL Prime Western Blotting Detection Reagent and detected with a ChemiDoc XRS+ system. Images were processed and quantified with Image Lab 3.0.

S.2.4 Statistical Analyses

The differences between the control and normal groups were tested with unpaired student t-test unless specified. The differences between the control groups and different dose treatment groups were tested with one-way analysis of variance (one-way ANOVA) by Dunnett's multiple comparisons test unless specified. All statistical analyses were performed at 5% level of significance ($p<0.05$) by using GraphPad Prism software version 6.01 (GraphPad Software, San Diego, California, USA). Data were expressed as mean ± standard deviation (SD) in all *in vitro* experiments.

S.3 Result

S.3.1 Measurement of cell proliferation and apoptosis by flow cytometry

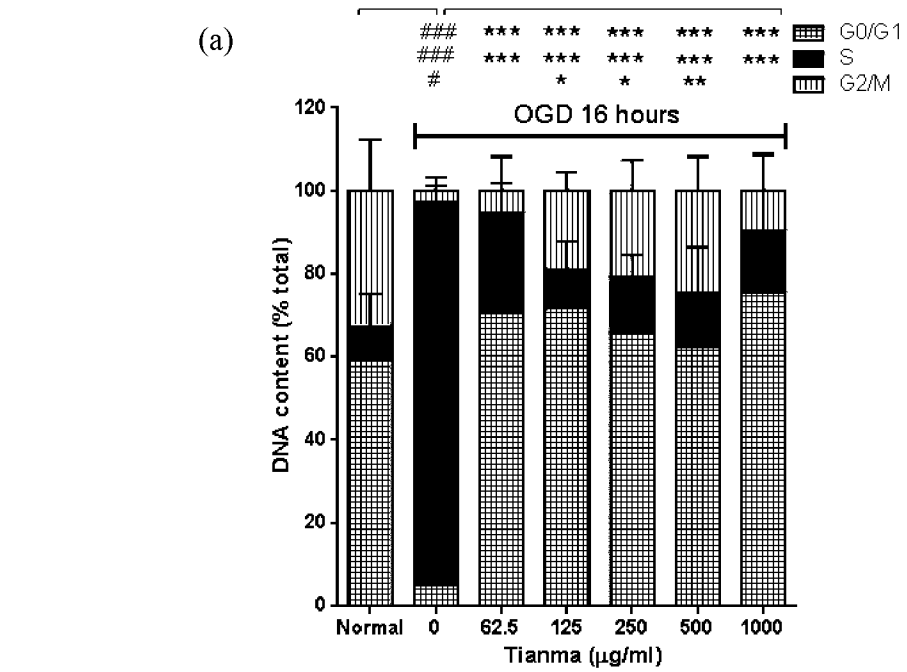
The effect of Tianma extract on overall cell cycle distribution on OGD-treated PC 12 cells were shown in Figure S.1a and S.1b. The stages of cell proliferation were divided into G_0/G_1, S and G_2/M phases.

The results for the cell cycle distribution after flow cytometry analysis were processed by the ModFit LT. The DNA content in the G_0/G_1 phase (Figure S.1c) of normal, control and Tianma treatment of 62.5, 125, 250, 500 and 1000 μg/mL were 59.2 ± 5.9%, 4.8 ± 3%, 70.7 ± 13.2%, 71.8 ± 6.2%, 65.6 ± 2.2%, 62.5 ± 2.7% and 75.4 ± 13.0%, respectively. The DNA content in G_0/G_1 phase in control after OGD treatment was significantly decreased ($p < 0.001$) when compared with the normal. The DNA contents after Tianma treatment (62.5, 125, 250, 500 and 1000 μg/mL) were increased significantly when compared with the control. A dose-dependent trend after Tianma treatment was not observed.

For S phase (Figure S.1d), DNA contents of normal, control and Tianma treatment of 62.5, 125, 250, 500 and 1000 μg/mL were 8.2 ± 7.8%, 92.3 ± 4.2%, 23.9 ± 13.7%, 9.2 ± 6.8%, 13.7 ± 5.2%, 13.0 ± 10.9% and 15.0 ± 18.5% respectively. The DNA content in S phase in control after OGD treatment was significantly increased ($p < 0.001$) when compared with the normal. The significant decrease of DNA contents after Tianma treatment (62.5, 125, 250, 500 and 1000 μg/mL) was observed in a dose-dependent manner.

For G_2/M phases (Figure S.1e), DNA contents of normal, control and Tianma treatment of 62.5, 125, 250, 500 and 1000 μg/mL were 32.6 ± 12.4%, 2.8 ± 3.2%, 5.4 ± 1.9%, 18.9 ± 4.6%, 20.7 ± 7.4%, 24.6 ± 8.3% and 9.6 ± 8.4% respectively. The OGD treatment significantly reduced ($p < 0.05$) the DNA content in the G_2/M phases in the control when compared with the normal. The Tianma extract of 125, 250 and

500 μg/mL could significantly reduce the DNA content ($p < 0.05$ for 125 and 250 μg/mL; $p < 0.01$ for 500 μg/mL) when compared with the control.



(b)

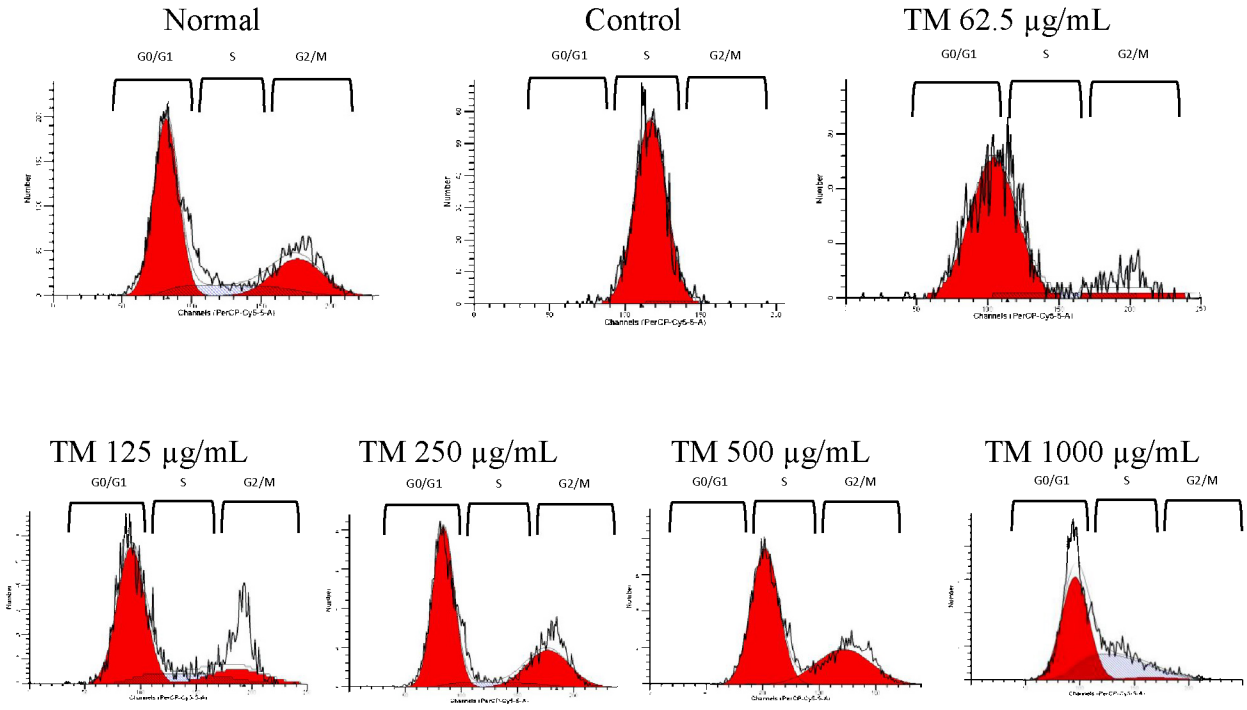


Figure S.1 Overall cell cycle distribution in PC 12 cells after OGD treatment (a) Relative DNA contents in different cell cycle phases. (b) Cell cycle distribution. Data are expressed as mean ± SD, n = 3 in 3 individual experiments. $^{###}p < 0.001$ as compared with normal group by Student's t-test. ***$p < 0.001$ as compared with control by one-way ANOVA.

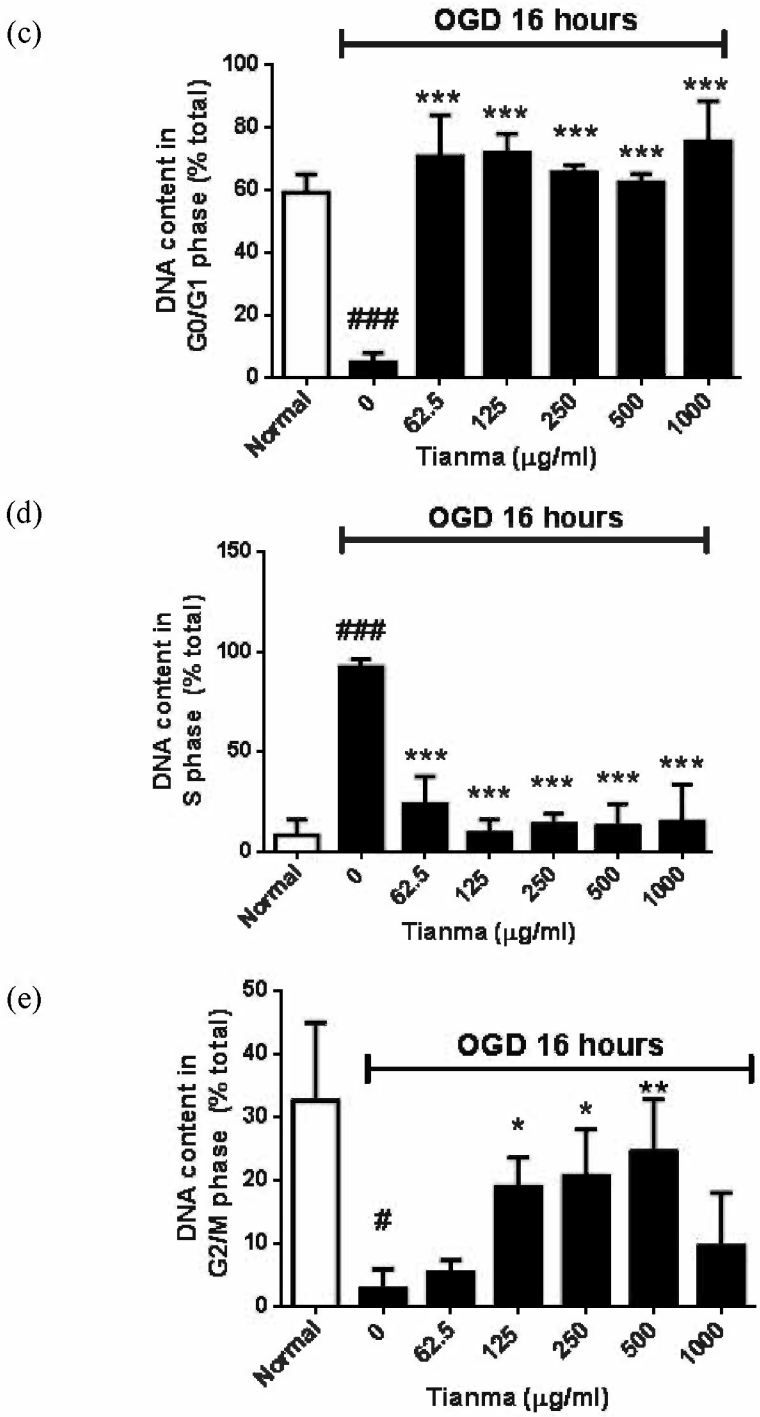
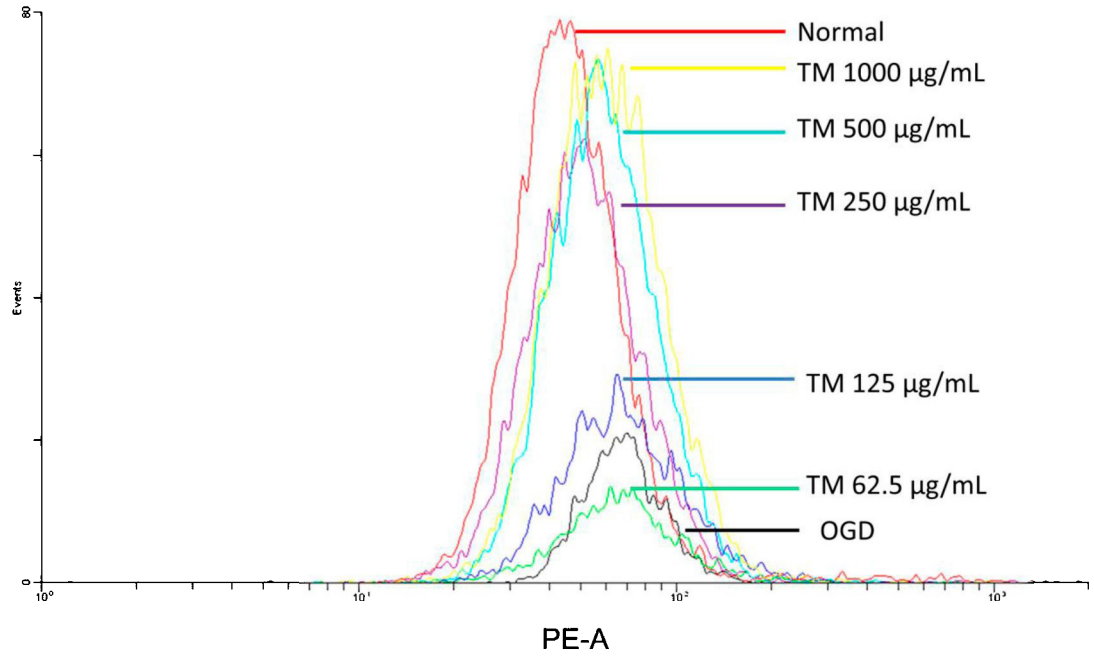


Figure S.1 Overall cell cycle distribution in PC 12 cells after OGD treatment (c) Relative DNA contents in G_0/G_1 phases. (d) Relative DNA contents in S phase. (e) Relative DNA contents in G_2/M phases. Data were expressed as mean ± SD, n = 3 in 3 individual experiment. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ as compared with normal group by Student's t-test. *p < 0.05, ** p < 0.01 as compared with control by one-way ANOVA.

The results for apoptosis measurement from flow cytometry analysis were processed. The flow diagram of cleaved PARP was shown in Figure S.2a. The right-shifted curve of cleaved PARP implied that more PC 12 cells underwent apoptosis after OGD treatment. Cells shifted to the left after Tianma treatment. The quantified cleaved PARP population of normal, control and Tianma treatment of 62.5, 125, 250, 500 and 1000 μg/mL were 49.9 ± 1%, 94.7 ± 1.5%, 87.1 ± 6.7%, 83.9 ± 7.1%, 71.5 ± 10.5%, 77.1 ± 7.1% and 76.4 ± 9.2% respectively, shown in Figure S.2b. The cleaved PARP population in the control was significantly increased ($p < 0.001$) when compared with the normal after OGD treatment. The cleaved PARP population after Tianma treatment was reduced in a dose-dependent manner. The Tianma extract of 250 and 1000 μg/mL could significantly reduce the cleaved PARP population ($p < 0.05$) when compared with the control.

(a)



(b)

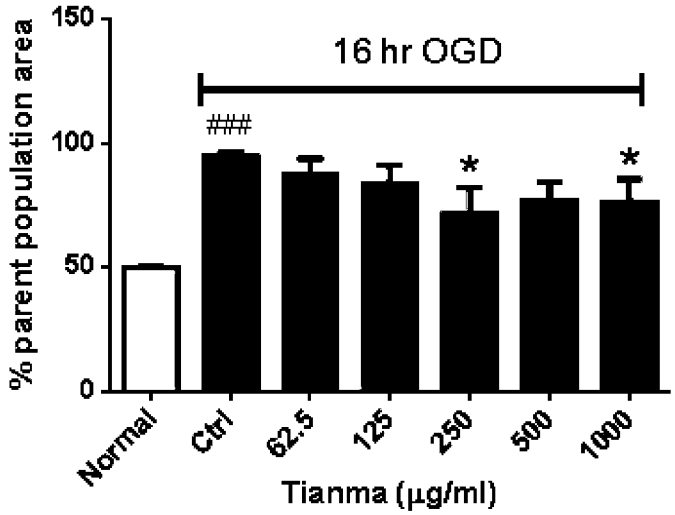


Figure S.2 Flow cytometric analysis of apoptosis by cleaved PARP measurement in Tianma-treated PC 12 cells after OGD treatment. (a) Flow diagram of cleaved PARP. Different colours indicated corresponding peak in the diagram. (b) Cleaved PARP population in PC 12 cells after OGD treatment. Data were expressed as mean ± SD, n = 3 in 3 individual experiment. $^{###}p < 0.001$ as compared with normal group by Student's t-test. *$p < 0.05$ as compared with control by one-way ANOVA.

S.3.2 Determination of anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax

The *in vitro* studies on the effect of Tianma extract on anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax expression after OGD-induced apoptosis on PC 12 cells were investigated by Western blotting (Figure S.3a).

The results on Bcl-2 expression were shown in Figure S.3b. The relative expression of Bcl-2 in whole cell extract of normal, control, Tianma treatment of 62.5, 125, 250, 500 and 1000 μg/mL, normalized with β-actin were 100 ± 0%, 5.0 ± 1.8%, 15.3 ± 2.4%, 36.4 ± 10.6%, 63.2 ± 13.6%, 71.9 ± 3.6% and 79.3 ± 3.6% respectively. Bcl-2 expression of the control group significantly decreased ($p < 0.001$) as compared with the normal group. A dose-dependent significant increase ($p < 0.001$) of Bcl-2 expression was observed after Tianma treatment ranged from 62.5, 125, 250, 500 and 1000 μg/mL.

The results on Bax expression were shown in Figure S.3c. The relative expression of Bax in whole cell extract of normal, control, Tianma treatment of 62.5, 125, 250, 500 and 1000 μg/mL, normalized with β-actin were 0.2 ± 0.2%, 100 ± 0%, 22.9 ± 9.8%, 9.6 ± 5.2%, 1.56 ± 1.1%, 0.8 ± 0.7% and 0.5 ± 0.6% respectively. Bax expression of the control group significantly increased ($p < 0.001$) as compared with the normal group. A dose-dependent decrease of Bax expression was observed after Tianma treatment. The Tianma extract ranged from 62.5, 125, 250, 500 and 1000 μg/mL could significantly reduce ($p < 0.001$) the Bax expression induced by OGD.

We also compared the Bcl-2/Bax expression ratio after analysis. The result was shown in Figure S.3d. The relative Bcl-2/Bax expression ratio in whole cell extract from PC 12 cells with the absence or presence of OGD and Tianma extract ranged from 62.5, 125, 250, 500 and 1000 μg/mL, normalized with β-actin were 100 ± 0%, 0.6 ± 0.2%, 1 ± 0.7%, 2.8 ± 1.9%, 13.3 ± 6.2%, 25.7 ± 10.8% and 41.2 ± 16.9%,

respectively. The Bcl-2/Bax expression ratio increased in a dose-dependent manner. The OGD treatment would significantly decrease ($p < 0.001$) the Bcl-2/Bax expression ratio. The ratios were significantly ($p < 0.001$) increased after treated by 500 and 1000 μg/mL Tianma extract.

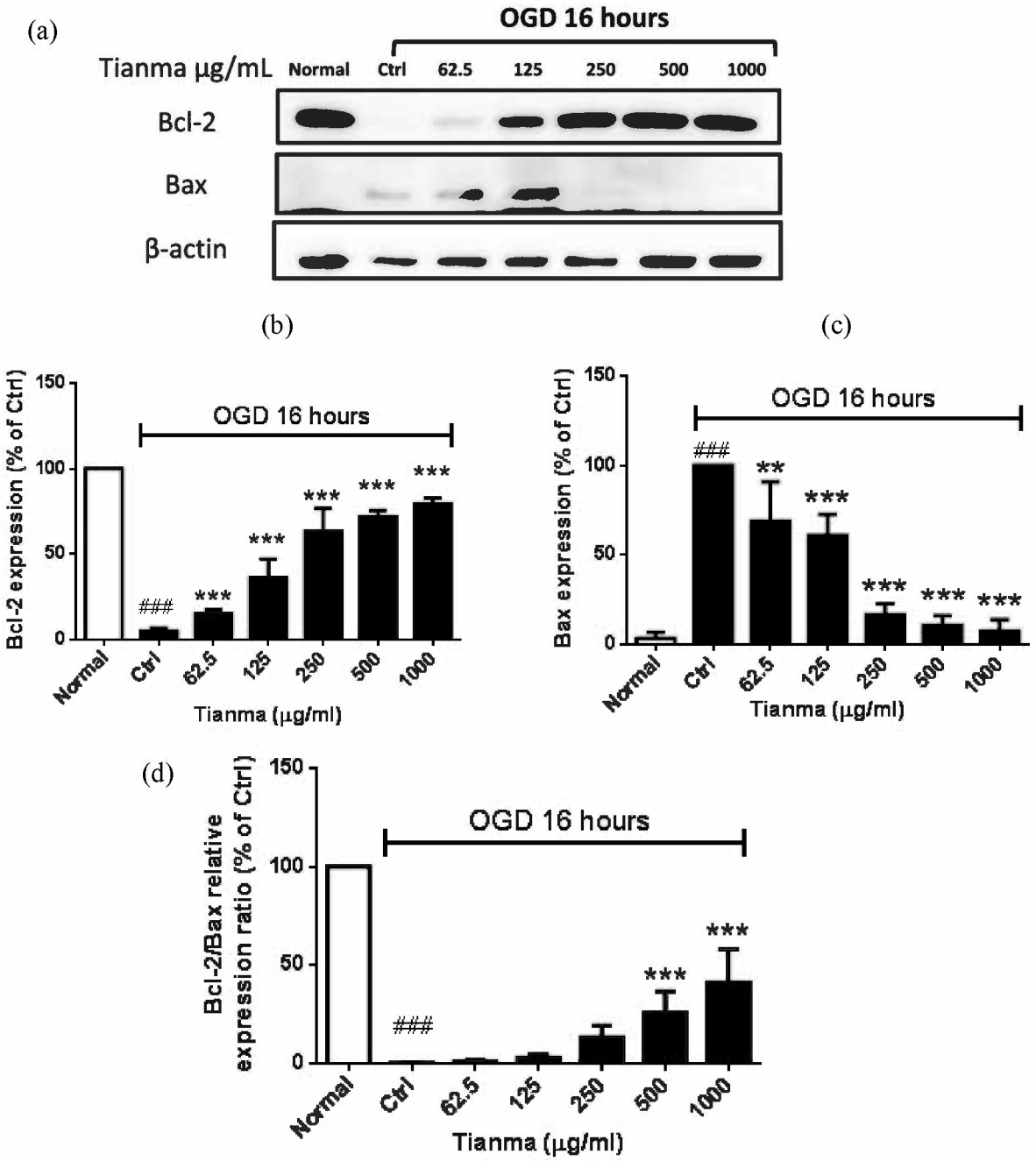


Figure S.3 Bcl-2 and Bax expression in PC 12 cells after OGD treatment. (a) Total protein extracts were prepared using RIPA lysis buffer and subjected to 10% SDS-PAGE to determine protein expression (b) Relative Bcl-2 expression (c) Relative Bax expression. (d) Bcl-2/Bax expression ratio. Data were expressed as mean ± SD, n = 4 in 4 individual experiments. $^{###}p < 0.001$ as compared with normal group by Student's t-test. ***p < 0.001 as compared with control by one-way ANOVA.

S.4 Discussion

In this chapter, we investigated the anti-apoptotic effects of Tianma water extract by using flow cytometry as well as the expression of two apoptotic proteins, anti-apoptotic Bcl-2 and pro-apoptotic Bax after OGD treatment.

We used OGD *in vitro* model on PC 12 cells for investigation of anti-apoptotic effects of Tianma water extract because numerous studies had that stated the apoptosis-related pathways were initiated after cells underwent OGD treatments. For example, caspase-3, a vital mediator in the apoptosis, was activated in the OGD treatment (Hillion *et al.*, 2005). Besides, the upregulation of pro-apoptotic Bax protein and downregulation of anti-apoptotic Bcl-2 protein were also observed (Song *et al.*, 2006).

The Apoptosis, DNA Damage and Cell Proliferation Kit used in our study was a relative new innovation which allow multicolor flow cytometric analysis of cell proliferation and apoptosis fluorescent antibodies by incorporating BrdU and cleaved PARP, respectively within one sample, according to manufacturer's protocol. The cell proliferation and apoptosis were our interest. The 5-bromo-2'-deoxyuridine (BrdU) stained cellular analysis by the flow cytometry enabled us to determine the cell-cyle distribution as the BrdU, the synthetic analog of the DNA precursor thymidine, which would incorporate into newly cellular synthesized DNA during cell replication (Dolbeare *et al.*, 1983).

Neuronal cell death has been proven to be related to interrupted cell cycle (Byrnes and Faden, 2007; Nguyen *et al.*, 2002). One of the apoptotic mechanisms was characterized by a process called "Cell cycle re-entry" which involved aberrant cell cycle proteins expression, especially in S phase. (Folch *et al.*, 2012).

The proliferation status of cells was reflected by the changes of DNA content in different phases (G_1, S, G_2 and M) (Resnitzky and Reed, 1995). G_1 phase is the

restriction point in the cell cycle for cell proliferation (Blagosklonny and Pardee, 2002). From the cell cycle distribution (Figure S.1), the OGD treatment significantly decreased the DNA content of control group in G_0/G_1 phase while a significant increase in DNA content in S phase was observed. This revealed that the cell was excited from the cell cycle and passed G_1/S checkpoint. However, cell death instead of cell division would be resulted from this abnormal cell cycle re-entry (Zhang *et al.*, 2009). After Tianma treatment, DNA content in G_0/G_1 phase increased while those in S phase attenuated, indicating more cells were excited from the cell cycle. We suggested this was probably due to the effect of Tianma water extract on modulating the cell cycle protein expression to prevent the cell cycle re-entry. This result was conherent with the result of anti-apoptotic effect of Tianma water extract in PARP cleavage study.

On the other hand, the OGD treatment significantly decreased the DNA content of control group in G_2/M phases. Once DNA synthesis was completed, cells would enter the G_2 phase and prepared for mitosis (M phase). Moreover, the G_2/M transition to new early G_1 was another cell proliferation critical point (Blagosklonny and Pardee, 2002; Olmeda *et al.*, 2003). The decrease of DNA content in this phase showed that the OGD inhibited the PC 12 cells proliferation by blocking the transition between late S phase to G_2 or even mediating arrest at the G_2/M cell cycle. Adenomatous polyposis coli (APC) played a critical role in the G_2/M phase. Once APC bound to proteins such as β-catenin, axin, etc., this complex would prevent the cell from re-entering the new G_1 phase (Aoki and Taketo, 2007; Olmeda *et al.*, 2003). We suggested the Tianma extract probably inhibited the formation of this complex and thus promoted the cell proliferation. However, further studies have to be done to verify these hypothesis.

In G_2/M phases, Tianma extract at 1000 μg/mL behaved as a outliner from the

dose-dependent trend. We suggested that at this high dose, some antagonistic effects among the ingredients within the extract might weaken its proliferation promotion effect.

Poly ADP-ribose polymerase (PARP) is a nuclear enzyme that binds rapidly to broken DNA for repair (Herceg and Wang, 2001; Lindahl *et al.*, 1995). Under normal condition, when DNA strand broke, NAD^+ would be recruited as substrate by PARP. ADP-ribose moieties would be transferred to nuclear acceptor proteins for DNA repair (Carson *et al.*, 1986). During apoptosis, the activation of caspase-3 and caspase-7 would lead to the cleavage of numerous substrate proteins (Endres *et al.*, 1997; Germain *et al.*, 1999; Namura *et al.*, 1998), including PARP. PARP was then cleaved into 89- (with a catalytic domain) and 24-kDa fragments (with DNA binding domain, DBD) (Soldani and Scovassi, 2002). Due to the presence of the catalytic domain, the 89-kDa fragment still carried basic enzyme activity but no longer to be stimulated by DNA strand breaks. As 89-kDa fragment became inactive to DNA damage occurred, NAD^+ recruitment was inhibited for DNA repair (Carson *et al.*, 1986). As the 24-kDa fragment had the DBD, it was able to bind with RNA and DNA irreversibly (Smulson *et al.*, 1998), just like uncleaved PARP but with lower affinity (Yung and Satoh, 2001). However, this binding affinity would inhibit the normal catalytic activity of the intact PARP, leading to a transdominant inhibition on synthesis of Poly ADP-ribose (D'Amours *et al.*, 2001) and the termination of removal of non-helix-distorting base lesions from the genome, known as "Base Excision Repair" (BER) (Liu *et al.*, 2007). These would finally destroy PARP DNA repair ability (Boulares *et al.*, 1999; Herceg and Wang, 2001).

Therefore, the cleaved PARP fragment was considered as a marker of cellular apoptosis as well as reflecting the caspase activities. This kit enabled us to investigate the anti-apoptotic effect of Tianma extract by measuring relative amount

of cleaved PARP fragment. Our result (Figure S.2) showed that the PARP fragmentation was significantly increased after OGD treatment. This finding was consistent with the apoptosis induced by OGD in a previous study (Hillion *et al.*, 2005). After Tianma treatment, the PARP cleavage was significantly reduced, implying the downregulation of the caspase. The anti-apoptotic effects of Tianma extract might be applied on the caspase regulation as PARP could be cleaved almost by all caspase *in vitro* (Duriez and Shah, 1997). The effects of Gastrodin on caspase-3 have been revealed that the Gastrodin could downregulate the caspase-3 *in vivo* (Bin, 2011) or *in vitro* (Tsai *et al.*, 2011) via different pathways. Similar effects may be carried by Tianma water extract but further studies were needed to verify.

As mentioned, intrinsic and extrinsic pathways are involved in series events in apoptosis. The intrinsic pathway is mitochondrial-dependent and involves the interactions and regulations of pro-apoptotic protein Bax and other anti-apoptotic Bcl-2 family members (Lopez-Neblina *et al.*, 2005). The expression of these mediators will activate caspases and affects the apoptosis.

The prologue of apoptosis is the release of cytochrome *c* from the mitochondria (Liu *et al.*, 1996). The formation of the Apaf-1-caspase-9-cytochrome c complex, after the entry of cytochrome *c* into the cytosol and with the presence of dATP or ATP (Li *et al.*, 1997; Vaux, 1997; Zou *et al.*, 1997), would trigger a caspases-activation cascade, especially towards caspases-3, caspases-6 and caspases-7 (Li *et al.*, 1997; Pan *et al.*, 1998). As a result, the activation of caspases would lead to the cleavage of numerous substrates and promoted apoptosis (Endres *et al.*, 1997; Germain *et al.*, 1999; Namura *et al.*, 1998).

B-cell lymphoma/leukemia-2 (Bcl-2) gene, known as a proto-oncogene, was discovered at the chromosomal breakpoint of t(14;18) bearing human B-cell lymphomas (Tsujimoto and Croce, 1986). Bcl-2 protein, a member of Bcl-2 gene

family, was localized at the inner mitochondrial membrane (Hockenbery *et al.*, 1990). It served as a repressor in the apoptosis (Oltval *et al.*, 1993) in that it could promote cell survival but independent of cell proliferation (Dohlman *et al.*, 1988). It could inhibit apoptosis by blocking the spontaneous release of cytochrome *c* from mitochondria (Kluck *et al.*, 1997; Yang *et al.*, 1997), thus prevented the activation of caspases (Chinnaiyan *et al.*, 1996; Shimizu *et al.*, 1996). Bcl-2-associated X protein (Bax) was a member protein belonging to the Bcl-2 gene family and was known to be pro-apoptotic (Lodish *et al.*, 2000). Under normal situation, Bax was localized in the cytosol. When apoptosis was initiated, Bax would undergo a conformation shift and translocate to the mitochondria, thus becoming mitochondrial membrane-associated (Hsu *et al.*, 1997; Wolter *et al.*, 1997). The pro-apoptotic feature of Bax was shown to be associated with the induction of cytochrome *c* release (Jürgensmeier *et al.*, 1998; Rossé *et al.*, 1998) and the disruption of the mitochondrial inner transmembrane potential (Pastorino *et al.*, 1998). This increased the release of cytochrome *c* and in turn led to caspase activation (Weng *et al.*, 2005). The Bax-induced apoptosis had been shown to be inhibited by Bcl-2 but not the Bax-induced cytochrome *c* release in cells (Rossé *et al.*, 1998), the expression ratio of Bcl-2/Bax was a prime factor to determine whether the cells survived or underwent apoptotic cell death (Korsmeyer *et al.*, 1993).

From the results of Bcl-2 and Bax expression after OGD treatment (Figure S.3), the expression of Bcl-2 decreased while Bax increased significantly in the control group. This showed that apoptosis was enhanced after the OGD treatment by the overexpression of Bax and was consistent with the increased cleaved PARP. After Tianma treatment, the expression of Bcl-2 was upregulated while Bax was downregulated significantly, so as to tilt the Bcl-2/Bax expression ratio upwards (Korsmeyer *et al.*, 1993). This indicated that the anti-apoptotic activities of Tianma

extract was due to its regulatory ability on the apoptotic proteins. This regulation might be via the A2A-R/ cAMP/ PKA/ CREB-dependent pathway as Tianma ethanol extract had been found to inhibit apoptosis by promoting cAMP formation, PKA activity and CREB phosphorylation (Tsai *et al.*, 2011). Similar regulatory mechanisms may be found in Tianma water extract. Another possible pathway might be the regulatory effects on the intracellular Ca^{2+} and glutamate-induced apoptosis as chemical components of Tianma, vanillin and 4-HBAL were all involved in this regulation (Lee *et al.*, 1999).

Some early studies also stated that the anti-apoptotic Bcl-2 might be an anti-oxidant (Hockenbery *et al.*, 1993; Jacobson and Raff, 1995; Kane *et al.*, 1993) apart from being a death-repressor (Hockenbery *et al.*, 1990) due to its localization to endoplasmic reticulum, nuclear membrane as well as cytoplasmic face of the mitochondrial outer membrane, which were the intracellular sites of ROS generation (Gotow *et al.*, 2000; Haldar *et al.*, 1994). This suggestion was supported by *in vitro* neuronal cell studies (Ellerby *et al.*, 1996; Myers *et al.*, 1995) and Bcl-2 knockout mice study (Veis *et al.*, 1993). However, only limited researches have been performed on this issue and no hypothesis or conclusion could be drawn as to whether the effects of Tianma water extract on upregulating Bcl-2 would exacerbate the anti-oxidative effect in our studies.

In conclusion, Tianma water extract was proven to improve cell cycle transition from phase to phase and prevent cell cycle re-entry. Moreover, our study also showed an anti-apoptotic effect by inhibition of PARP cleavage, probably related to the caspase-3 inactivation and downregulation of pro-apoptotic protein Bax, as well as upregulation of anti-apoptotic Bcl-2. Besides the typical apoptotic pathways of tissue damage, ROS and NO were proven in previous studies that they were acting as mediators to regulate the necrotic and apoptotic cell death after ischemic stroke

(Buttke and Sandstrom, 1994; Chopp *et al.*, 1996; Stamler *et al.*, 1992). The anti-oxidative and anti-inflammatory effects of Tianma water extract eventually might have led to the anti-apoptotic effects of Tianma water extract. These effects might account for the amelioration of ischemic infarct of MCAo rat model. These findings establish the therapeutic possibilities of using Tianma water extract for ischemic stroke associated apoptotic injuries. However, further studies are required to confirm the precise mechanisms of Tianma extract Tianma extract for ischemic stroke treatment.

S.5 Limitation of the anti-apoptotic effect of Tianma water extract study and future work

The anti-apoptotic effect Tianma water extract was proven by modulation on cell cycle transition, reduction of cleaved PARP and regulation on Bcl-2 and Bax. However, data in cell cycle study only provided a peripheral picture on the effect of Tianma water extract on cell cycle distribution. Future work should be done on the the expression on different cell cycle proteins such as p53, cyclin-dependent protein kinases CDK4 and CDK6, etc. Futhermore, studies on the caspase-3 activities should be performed to investigate if the reduced PARP cleavage was due to the downregulation of caspase-3 activities by Tianma extract. Moreover, cytochrome *c* was an important mediator in apoptosis. Further studies should be done to investigate whether Tianma extract could downregulate the cytochrome *c* expression.

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