# The Neuro-protective Effects of Curcumin Against I/R Injury in Transient MCAO Rat Model

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# ผลของเคอร์คูมินต่อการป้องกันสมองจากการบาดเจ็บจาก I/R
# ในโมเดลของหนูทดลองที่ทำให้เกิด MCAO แบบชั่วคราว

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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เหว่ย ลี : ผลของเคอร์คูมินต่อการป้องกันสมองจากการบาดเจ็บจาก I/R ในโมเดลของหนูทดลองที่ทำให้เกิด MCAO แบบชั่วคราว (The Neuro-protective Effects of Curcumin Against I/R Injury in Transient MCAO Rat Model) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.สุทธิลักษณ์ ปทุมราช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ.นิจศรี ชาญณรงค์, 150 หน้า.

การเกิดออกซิเดชัน การอักเสบ และการเกิดอะโพโทซิส เป็นสามปัจจัยที่ร้ายแรงที่ทำให้เกิด การสูญเสียหน้าที่เมื่อมีการเกิดภาวะที่สมองขาดเลือดไปเลี้ยงและเมื่อมีการนำเลือดกลับไปเลี้ยง ตามที่มีรายงานแสดงว่าเคอร์คูมินมีคุณสมบัติที่สามารถต้านการเกิดออกซิเดชัน ต้านการอักเสบ และต้านการเกิดอะโพโทซิส แต่อย่างไรก็ดีกลไกการทำงานในระดับโลเลกุลในการเกิดการบาดเจ็บเมื่อมีการเกิดภาวะที่สมองขาดเลือดไปเลี้ยงและเมื่อมีการนำเลือดกลับไปเลี้ยงในสมองนั้นยังไม่ทราบแน่ชัด ในงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อ ศึกษาหากลไกของเคอร์คูมินต่อปริมาณ การบวมน้ำ การตายของเนื้อสมอง การสูญเสียหน้าที่ของผนังหลอดเลือดที่กั้นระหว่างเลือดและสมอง และการเกิดการแสดงออกของอินเตอร์เซลลูลาร์แอดฮีซันโมเลกุล-1 เมตริกซเมทัลโลโปรตีเนส-9 เอนไซม์ แคสเปส-3 นิวเคลียร์แฟคเตอร์แคปปาบี และ เอ็นอารเอฟ-ทู โดยทำการศึกษาในโมเดลของหนูแร็ทเพศผู้ ที่ถูกทำให้เกิดการอุดกั้นหลอดเลือดแดงในสมองส่วนกลาง (MCAO โดยทำการอุดกั้นการไหลของเลือดเป็นเวลา 1 ชั่วโมง แล้วจึงนำเลือดกลับไปเลี้ยงอีกเป็นเวลา 24 ชั่วโมง) สำหรับหนูกลุ่ม MCAO+CUR หนูกลุ่มนี้จะได้รับการฉีดสารละลาย เคอร์คูมินขนาด 300มก.ต่อกก. น้ำหนักตัว ทางช่องท้อง ที่ช่วงเวลา 30 นาทีหลังการอุดกั้น ส่วนในหนูกลุ่ม SHAM จะเป็นหนูที่ได้ทำการผ่าตัดทุกอย่างแบบเดียวกันยกเว้นแต่ไม่มีการอุดกั้น เมื่อครบ 24 ชั่วโมงหลังการนำเลือดกลับไปเลี้ยง จึงทำการวัดหาค่าพารามิเตอร์ต่างๆคือ การบวมน้ำ การตายของเนื้อสมอง การสูญเสียหน้าที่ของผนังหลอดเลือดที่กั้นระหว่างเลือดและสมอง แล้วจึงทำการเก็บตัวอย่างเนื้อสมองเพื่อนำไปหา การเกิดการแสดงออกของอินเตอร์เซลลูลาร์แอดฮีซันโมเลกุล-1 เมตริกซเมทัลโลโปรตีเนส-9 เอนไซม์ แคสเปส-3 นิวเคลียร์แฟคเตอร์แคปปาบี และ เอ็นอารเอฟ-ทู โดยวิธีอิมมูโนฮีสโตเคม เมื่อทำการเปรียบเทียบผลพบว่า ในกลุ่ม MCAO มีการบวมน้ำ การตายของเนื้อสมอง การสูญเสียหน้าที่ของผนังหลอดเลือดที่กั้นระหว่างเลือดและสมอง และมีการแสดงออกของอินเตอร์เซลลูลาร์แอดฮีซันโมเลกุล-1 เมตริกซเมทัลโลโปรตีเนส-9 เอนไซม์ แคสเปส-3 นิวเคลียร์แฟคเตอร์แคปปาบี มากกว่ากลุ่ม SHAM แต่มีการแสดงออกของ เอ็นอารเอฟ-ทู น้อยกว่ากลุ่ม SHAM อย่างมีนัยสำคัญทางสถิติ ผลการทดลองแสดงให้เห็นว่า เคอร์คูมิน มีฤทธิ์ในการป้องกันการสูญเสียหน้าที่ในภาวะ ที่สมองเกิดการอุดกั้นหลอดเลือดแดงและมีการนำเลือดกลับไปเลี้ยง โดยอาศัยกลไกการ ต้านการเกิดออกซิเดชัน ต้านการอักเสบ และต้านการเกิดอะโพโทซิส และเกี่ยวข้องกับฤทธิ์ของเคอร์คูมิน ที่สามารถเพิ่ม เอ็นอารเอฟ-ทู แต่มีฤทธิ์ในการยับยั้งการแสดงออกของอินเตอร์เซลลูลาร์แอดฮีซันโมเลกุล-1 เมตริกซเมทัลโลโปรตีเนส-9 เอนไซม์ นิวเคลียร์แฟคเตอร์แคปปาบี และ แคสเปส-3

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Oxidation, inflammation, and apoptosis are three critical factors for the pathogenic mechanism of cerebral ischemia/reperfusion (I/R) damage. Curcumin has been elucidated to exhibit substantial biological properties via anti-oxidation, anti-inflammation and anti-apoptosis effects, however, its molecular mechanism against cerebral I/R injury remains unclear. To investigate the effects of curcumin on cerebral I/R injury associated with water content, infarction volume, blood-brain barrier (BBB) disruption and the expression of ICAM-1, MMP-9, NF-kappa-B, caspases-3, and Nrf2. The middle cerebral artery occlusion (MCAO, 1- hour occlusion and 24-hour reperfusion) was performed in male Wistar rats as representing cerebral I/R injury model. In MCAO+CUR group, rats were received curcumin administration (300 mg/kg BW, ip.) at 30-min after occlusion. The same operated procedures were performed in SHAM rats without MCAO occlusion. At 24-hour post-operation, all of these parameters including neurological deficit scores, BBB disruption, water content, and infarction volume were determined. Brain tissue ICAM-1, MMP-9, NF-kappa-B, caspases-3, and Nrf2 were assayed by immunohistochemistry. Compared with SHAM group, the BBB disruption, neurological deficit scores, brain water content and infarction volume were severely demonstrated in MCAO group. ICAM-1, MMP-9, NF-kappa-B and caspases-3 were enhanced in MCAO group. However, in MCAO+CUR group, the upregulated Nrf2, an anti-oxidation related protein, collaborating with the decline of other biomarkers were significantly observed. The protective effects of curcumin against cerebral I/R injury were attributed to its anti-oxidation, anti-inflammation and anti-apoptosis which were involved in the up-regulation of Nrf2 and the down-regulation of ICAM-1, MMP-9, NF-kappa-B and caspases-3.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| WHO | World Health Organization |
| I/R | ischemia/reperfusion |
| NF-κB | nuclear factor-κB |
| BBB | blood brain barrier |
| MMPs | matrix metalloproteinase |
| Nrf2 | transcription factor nuclear factor-erythroid-2-related factor 2 |
| MCAO | middle cerebral artery occlusion |
| IKK | IκB kinase |
| ICAM-1 | intercellular adhesion molecule-1 |
| Ecs | endothelial cells |
| TIMPs | tissue inhibitor of metalloproteases |
| Apaf-1 | apoptosis protease activating factor-1 |
| ROS | reactive oxygen species |
| RNS | reactive nitrogen species |
| ZO-1 | zonula occludens-1 |
| nNOS | neuronal nitric oxide synthases |
| iNOS | inducible NOS |
| Keap1 | kelch-like erythroid cell-derived protein with CNC homology associated protein 1 |
| ARE | antioxidant response element |
| FDA | Food and Drugs Administration |
| BDMC | bisdemethoxycurcumin |
| DMC | demethoxycurcumin |
| CCA | common carotid artery |
| ECA | external carotid artery |
| ICA | internal carotid artery |
| CBF | cerebral blood flow assay |
| LDF | Laser-Doppler perfusion monitor |

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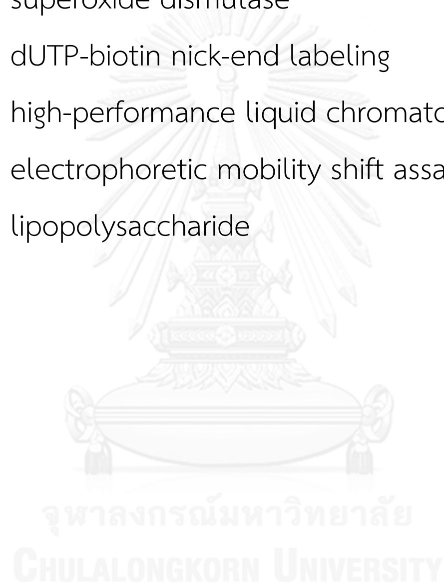
|---|---|
| FITC-dextran-250 | fluorescence isothiocyanate-labeled dextran-250 |
| aCSF | artificial cerebral spinal fluid |
| MDA | malondialdehyde |
| IOD | integrated optical density |
| SEM | standard error of mean |
| ANOVA | one-way analysis of variance |
| MABP | mean artery blood pressure |
| H&E | hematoxylin and eosin |
| AQP | aquaporins |
| SOD | superoxide dismutase |
| TUNEL | dUTP-biotin nick-end labeling |
| HPLC | high-performance liquid chromatography |
| EMSA | electrophoretic mobility shift assay |
| LPS | lipopolysaccharide |



# CHAPTER I

## INTRODUCTION

### 1.1 Rationale

Stroke is the second cause of mortality and leading cause of disability for adult world wide [1, 2]. Over two-thirds of deaths due to stroke occur in developing countries [3]. Stroke can be divided into ischemic and hemorrhagic stroke according to the World Health Organization (WHO). Ischemic stroke accounts for approximately 85% of all stroke cases [4, 5], mostly result from occlusion of cerebral artery by embolism or thrombus, and the consequent of blood flow can block the nutrition and oxygen delivered to related brain, and lead to cerebral cell death and brain function invalidity [4].

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The pathophysiological mechanism behind cerebral ischemia/reperfusion (I/R) is as following [6], within several minutes, blocked cerebral blood vessel results in certain brain area hypo-perfusion, which can promptly induce brain cell death in the ischemic core area [7]. There is a penumbra area surrounding the core area, where cells are dysfunctioning but still viable because of collateral blood supply [8]. Prolonged ischemia can finally transform penumbra to dead tissue area. Cell death can be divided into necrosis and apoptosis depending on the severity and duration of damage to cell. Necrosis normally occurs in gusty, permanent vascular blockage,

whereas, reversible ischemia with reperfusion tends to milder damage, apoptosis are more prominent, especially within ischemic penumbra area [6].

In addition, hypoperfusion trigger production of free radicals and inflammatory responses. Through activation of nuclear factor-κB (NF-κB) pathway and unleashed enzymes, free radicals and inflammation can affect each other [9, 10], both of which can enhance cell death process and disrupt the blood brain barrier (BBB) integrity through activation of matrix metalloproteinase (MMPs) [11-13]. Simultaneously, NF-κB is able to directly contribute to apoptosis related neuronal cell death [14]. Promptly recirculation of the blocked blood vessel is an only clinical approved approach to rescue penumbra area by restoring oxygen and nutrition delivery. However, intravenous thrombolysis has limited 3.5-4 hours treatment window, many patients might not reach hospital within that short time period. Furthermore, reperfusion might enhance adverse risky of hemorrhagic transformation, cerebral edema and further cerebral cell death, especially delayed reperfusion [15].

In order to decrease reperfusion damage and optimize recanalization therapeutic effect, accumulating researches focus on the biomarkers of stroke pathophysiological pathways and explore ample neuro-protective agents, which performed successful intervention effects in animal experiments, but failed in randomized clinical trials with no exception [16]. Therefore, currently researchers pay

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more attention to daily dietary phytoalexin, especially multi-target agents on pathophysiological cascades of stroke.

As food and medicine widely used in many Asian countries, curcumin is a phenolic pigment extracted from rhizome of *Curcuma Longa* Linn [17]. Curcumin is a multi-functional material with a variety of cellular effects for different diseases [18]. Cumulative studies have reported that curcumin has strong neuroprotective effect on neuroinjury ailments [18] with no toxicity even at high dose [19].

The neuro-protective effects of curcumin against cerebral ischemia/reperfusion (I/R) damage are achieved mainly through restoring Blood brain barrier (BBB) disruption [20], decreasing cerebral infarction volume [21], ameliorating brain edema [22] and improving neurological deficit motor function [23] via anti-oxidant, anti-inflammation and anti-apoptosis [24, 25]. The better efficacy was acquired particularly when it is administered peripherally at high doses than oral usage [23].

The protective effects of curcuminoid treatment on the BBB disruption have been addressed in many studies by Evans blue detection [26]. However, the studies that was designed to check the BBB disruption by using intra-vital confocal microscopy was rare, especially at 24 h post reperfusion, which is the peak stage of BBB opening upon cerebral I/R [27]. Therefore, in this study, the benefit effects of

curcumin was emphasized on BBB disruption by intra-vital confocal microscopy detection at 24 hours post reperfusion.

In addition, in this study the anti-oxidant effect of curcumin was examined in detail whether it can up-regulate transcription factor nuclear factor-erythroid-2-related factor 2 (Nrf2) in cerebral I/R injury model or not. Since Nrf2 decreased in permanent MCAO rats model has been reported [28]. Up to our knowledge, very few studies have investigated the molecular mechanisms of curcumin effects on transient stroke model.

Matrix metalloproteinase (MMP) is one of the common enzymes which is associated with the BBB disruption upon the cerebral I/R injury. The anti-MMP property of curcumin has been identified in tumor disease [29]. In addition, as potent regulator, curcumin could perform its protective function to regulate NF-κB related proliferation, invasion, angiogenesis and apoptosis in many diseases [30-38]. However, for stroke, the effect of curcumin on NF-κB still remains great controversial.

Under cerebral I/R scenario, upon the stimulation from inflammation and/or oxidative stress, the p50 and p65 (RelA) subunits of NF-κB will be moved to the nucleus, to induce synthesis of proteins. Which is normally related to oxidative stress, inflammation and apoptosis, such as, TNF-α, IL-1β, ICAM-1, MMP-9, iNOS, eNOS, Bcl-2 and caspase-3 production [39]. The inhibition effect of curcuimin on NF-

κB activation and subsequent intercellular adhesion molecule 1 (ICAM-1) gene expression have been demonstrated in TNF-α stimulated human brain microvascular endothelial cell (HBMVEC) [40]. However, very few researches reported the effects of curcumin on MMP and NF-κB activation in cerebral I/R rat model.

Under these literature reviews, our novel usage of transient stroke rat model aimed to study the effects of curcumin on cerebral I/R injury in association with ICAM-1, MMPs, caspase-3, NF-κB, and Nrf2 expression.

## 1.2 Research questions

1. Can curcumin regulate ICAM-1, MMP-9, caspase-3, NF-κB and Nrf2 expression in transient MCAO rat model?
2. Can curcumin decrease neurological dysfunction, infarction volume, BBB integrity and edema against I/R injury in transient MCAO rat model?

## 1.3 Research objectives

1. To study the effect of curcumin on the expression of MMP-9, ICAM-1, caspase-3, NF-κB and Nrf2 in transient MCAO rat model.

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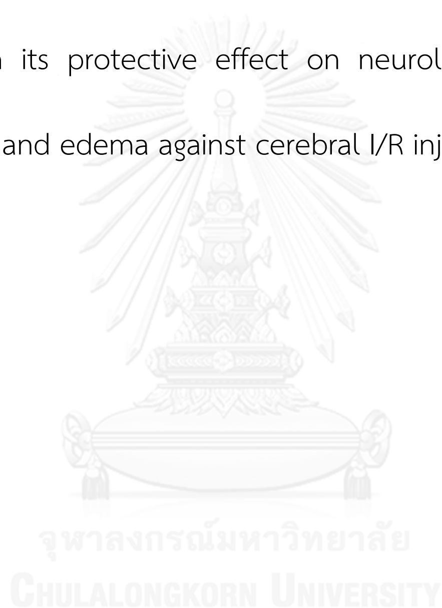
2. To study the effect of curcumin on the neurological dysfunction, infarction volume, BBB integrity and edema in transient MCAO rat model.

## 1.4 Hypothesis

Curcumin can regulate the expression of ICAM-1, MMP-9, caspase-3, NF-κB and Nrf2 to perform its protective effect on neurological dysfunction, infarction volume, BBB integrity and edema against cerebral I/R injury.



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1.5 Conceptual framework

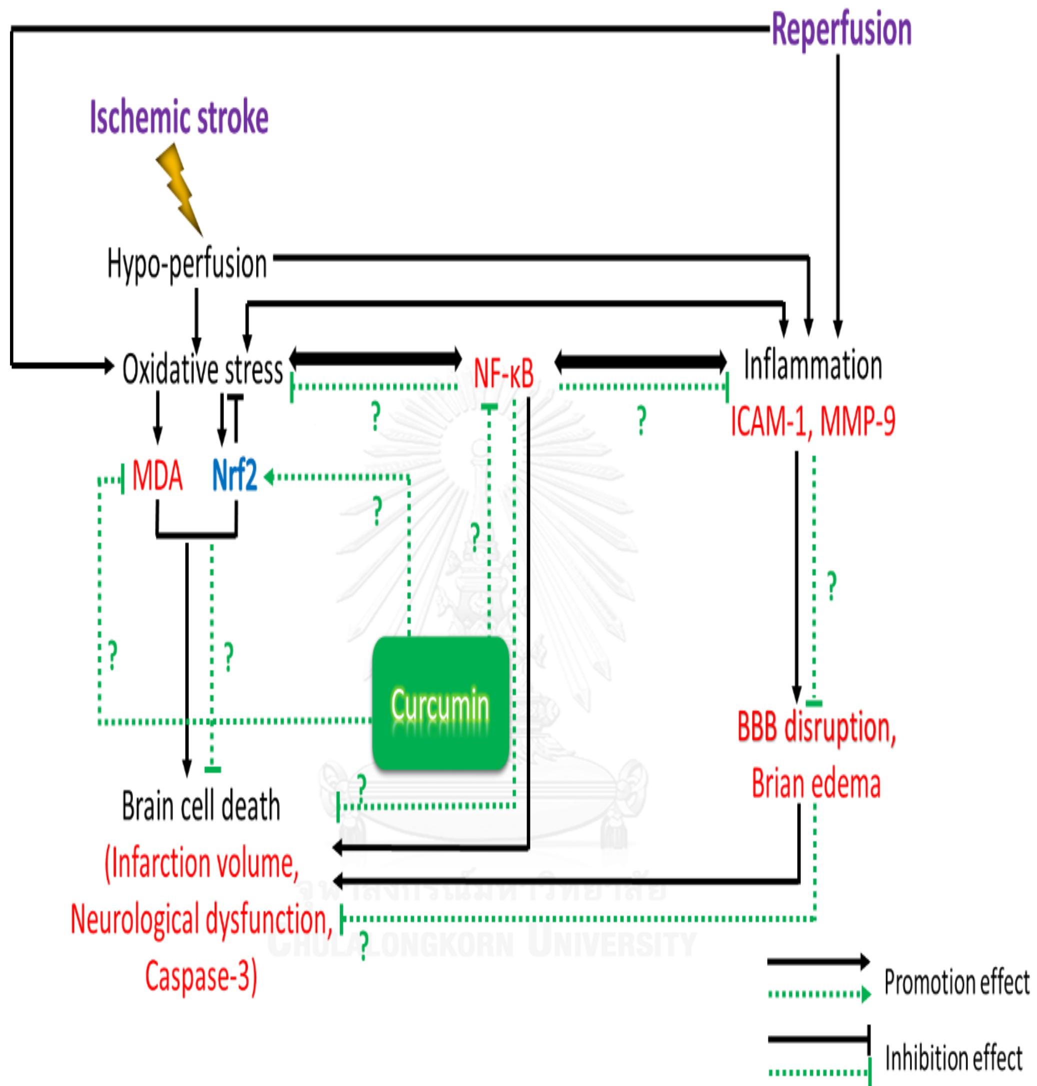


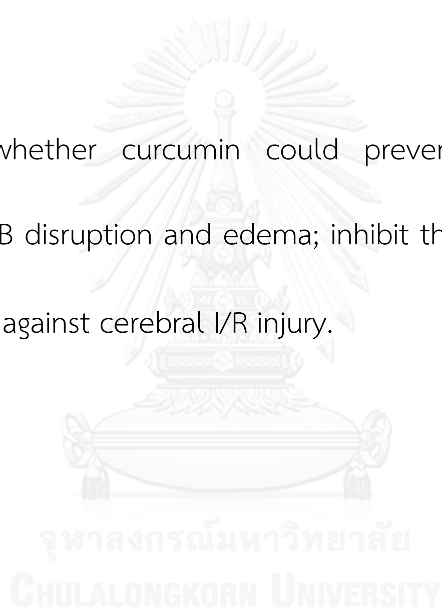

*Figure 1.1 Conceptual framework. The study framework is to investigate whether curcumin can regulate the expressions of ICAM-1, MMP-9, caspase-3, NF-κB and Nrf2 or not. And it may result in the protective effects of curcumin on neurological*

*function, brain infarction volume, BBB integrity, and brain water content against cerebral I/R injury.*

Figure key:

To be confirmed whether curcumin could help increase the activation of Nrf2 expression against cerebral I/R injury.

To be confirmed whether curcumin could prevent neurological dysfunction, infarction volume, BBB disruption and edema; inhibit the increase of ICAM-1, MMP-9, caspase-3 and NF-κB against cerebral I/R injury.



# CHAPTER 2

## LITERATURE REVIEW

### 2.1 Epidemiology of stroke

For adults, stroke is the leading cause of disability, and second cause of mortality worldwide [1]. Without any interference, the number of stroke deaths are predicted to be 6.5 and 7.8 million in 2015 and 2030, respectively. Within 15 years, the death rate will increase to 35.4% [41]. And over two thirds of stroke deaths occur in developing countries [42]. According to the World Health Organization, stroke ranks as the first leading cause of mortality in 2005 for Thailand [43]. The prevalence of stroke is predicted to be 1.88% for adults 45 years and older. There are more than 250,000 new stroke cases, and 50,000 lives are deprived from it in Thailand annually [5, 44-46].

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Ministry of Public Health of China reported in China Health Statistical Yearbook of 2013, that for rural residents, the stroke is the second leading cause of mortality; for urban citizens, is the third major cause of mortality. The overall stroke incidence is slightly higher in Chinese than white people. [5, 44, 45, 47]. Stroke lethally threatens the human’s health and life expectancy and tremendously consumes social sources and financial cost of health care, as the growing of aging,

obesity, and diabetes population, especially in low and middle income developing countries.

2.2 The classification of stroke

According to WHO, the definition of stroke is that, “rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin” [48]. It can be explained in a simple way that, when the blood flow to certain brain area is obstructed, which could result in corresponding neurological dsyfunction. it is called stroke. It is categorized into hemorrhagic or ischemic cerebrovascular accident based on the pathological background. The former originates from cerebral blood vessel rupture. And ischemic stroke results from the loss of blood flow to the certain brain region due to cardiac arrest (global ischemia) or blockage of certain cerebral artery (focal ischemia). For focal cerebral ischemia, it is normally caused by either embolism or thrombus to the cerebral artery, which is the most common stroke type, accounting for around 85% of all cases [4, 5].

According to Kanekar, the ischemic stroke could be divided into 4 stages, including hyper-acute, acute, sub-acute, and chronic periods on account of pathological course [41]. Hyper-acute stage is the optimized period of therapeutic intervention for ischemic stroke, it refers to the first 12 hours from the onset of

ischemia. The acute stage means 12-24 hours after stroke onset (elucidation below). The sub-acute stage starts from 2 days to 2 weeks post ischemia, which is characterized by cerebral edema and hemorrhagic transformation [41]. The chronic stage lasts from 2 weeks to 2 months after infarct, even though the effect may be sustained for years. The feature of this phase is involved in brain atrophy, gliosis, cavitations and calcifications with an augment of Wallerian degeneration and necrosis [41, 42].

### 2.3 Blood-brain barrier (BBB)

Cerebral capillary endothelial cells (ECs) and pericytes are encircled by a layer of basal lamina, which are encompassed by astrocytic end-feet, and through synapses, neighboring neurons could connect to the whole structure to form an entire functional unit called the neurovascular unit [49]. This unit functions as shield between blood circulation and brain parenchyma to confine the access of blood-originated substances and metabolism particles to the brain. Therefore it is also called BBB. In the latest years, much attention has been paid to BBB as a potential treatable target for cerebral I/R [50].

#### 2.3.1 Endothelial cells (ECs)

Compare with other periphery tissues, the ECs of this unit possesses several unique characters, for instance, (i) short of fenestrations, (ii) equipped with

tight junctions, (iii) have limited pinocytotic vesicles, (iv) contain a variety of enzymes able to decompose noxious substances and drugs, (v) facilitated with a great deal of mitochondria and polarized expression of membrane receptors and transporters, which provides sufficient energy for nutrients active transportation [51, 52]. Thus under physiological circumstances, the brain endothelia cells as the first defense line facing periphery circulation to protect the brain function.

2.3.2 Pericytes

Pericytes could control capillary blood perfusion through its vascular smooth muscle contractile property. In a mouse stroke experiment, the cerebral pericytes was observed contracted by the oxidative stress stimuli, which interfered capillary blood perfusion [53].

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2.3.3 Astrocytes

According to the literature review, the protective effect of astrocyte was found that it could release sonic hedgehog protein, which could stabilize the integrity of BBB [54, 55]. In addition, the anti-oxidants (glutathione and SOD) could be transferred to neurons by astrocytes to improve neuronal survival [56]. Whereas, upon cerebral I/R, the glutamate could cause lasting excitotoxicity to the neurons due to the uptake function of astrocytes is impaired. [57, 58].

### 2.3.4 Basal lamina

The basal lamina works as scaffold to lock the endothelium through the interaction between matrix proteins and ECs integrin receptors [52]. The basal laminal is formed by 3 layers, the main components of all three layers include glycoproteins, proteoglycans and collagen [59]. Many proteins involved in the regulation of BBB integrity via modulation of basal lamina proteins. Among them, the MMPs and their inhibitors, the tissue inhibitor of metalloproteases (TIMPs), play the most critical role in controlling the BBB permeability under physiological and inflammatory states [60].

### 2.3.5 Neurons

Neuron cells closely contact with the other cells of BBB through its projection, which can validly regulate the haemodynamics of blood perfusion by secretion of neuronal mediators.

## 2.4 Temporal characteristics of BBB disruption

After reperfusion, the BBB impairment progress in hyperemia stage followed by biphasic hypoperfusion stages [61]. The hyperemia stage last several minutes, associated with sudden increased of re-circulatory blood flow. The hypoperfusion stages, namely no flow phases, is divided into two phases. The former one has been

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associated with several factors, including lasting metabolic collapse, endothelial and astrocytes end-feet swelling and endothelial microvilli. In addition, the elevated leukocytes adhesion also make contribution to this stage. This episode is reversible phase, occurs at 3-8 hours post of reperfusion [61]. Besides the above factors, proteinase activation also involved, which related to the activation of MMP-2 [62]. MMP2 is constitutively expressed in latent form, normally found in CSF and astrocytes, which is also called gelatinase A. The last episode of BBB disruption starts from 18 h to 96 h after reperfusion, results in irreversible damage to the BBB integrity. The related factors involved in inflammation and oxidation. The executive factor is the activation of inducible MMP-9, namely, gelatinase B [62], which could directly destroy the basal laminal and tight junction of BBB.

2.5 Edema

As one of the critical consequence of BBB opening, edema could cause herniation to induce clinical deterioration [63]. Edema is classified into two types upon cerebral I/R, which are cytotoxic and vasogenic edema. Cytotoxic edema, which is associated with hyperemia after reperfusion. Imbalance of ionic exchange and dysregulation of metabolism leads to interstitial water translocate to intracellular compartment [64]. However, this stage will not increase the whole volume of brain, due to the cellular water intake result from the reduction of extracellular space [65]. Therefore the consequence from this stage is mild compare with vasogenic edema.

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Vasogenic edema results from the alteration of tight junction of BBB, relating to hypoperfusion stage post reperfusion. Owing to the increase of BBB permeability and osmosis, water could pass through the barrier accompany with blood component extravasation to the parenchyma, which could trigger much severe damage through enhancement of intracranial hypertension [65].

2.6 Mechanism of cerebral I/R on the acute stage

Within several minutes, blocked cerebral blood vessel induces hypo-perfusion in certain brain area, which can quickly cause energy failure, glutamate release, calcium overload inside of cell, and mitochondrial dysfunction [66]. Then free radicals and inflammatory responses are aroused, ultimately trigger brain cells death [67]. The damge area with irreversible pathological injury called ischemic core area [7]. There is a penumbra area surrounding the core area, where cells are dysfunction but still viable due to collateral blood supply [8]. Sustaining ischemia state can finally transform penumbra to be ischemic core region. Hence, promptly restore the flow to the ischemic area could rescue penumbra area. However, secondary damage could be induced by reflow process, especially delayed reflow. Potential secondary damage can amplify the production of free radicals and inflammation responses.

2.6.1 Promotion of brain cell death.

Brain cell death can be divided into necrosis and apoptosis, which depends on the severity and duration of damage to cell. Necrosis normally occurs in gusty, permanent vascular blockage, whereas, reversible ischemia with reperfusion tends to milder damage. Apoptosis is of more prominent, especially within ischemic penumbra area [6]. The neurons in the center of infarction area, are massively subjected to necrosis, characterized by swelling and rupture of organelles. For transient ischemic stroke, apoptosis is more prevailing, especially within ischemic penumbra area [6], which is the place that therapeutic intervention could rescue. Apoptosis is consequence of programmed cell death almost without inflammatory reaction compare with necrosis, The morphology of apoptotic cell is shrinkage of cytoplasm and nuclear, apoptotic body presence in the cytoplasm and formation of DNA fragments [68]. The apoptosis is highly conservative process, the same pattern of morphological feature happened in all of the multicellular organisms [69]. It is the process that the cell is organized to actively commit suicide, which is governed by a series of proteins activation. Bcl-2 family and caspase family are essential proteins to control process and progression of apoptosis activation [70]. The involved cells could be neurons, astrocytes, oligodendroglia, and inflammatory cells. It could be detected from hours to weeks after the injury onset [69].

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The classical apoptosis pathways could be categorized into caspase-dependent and caspase-independent ways. Caspase-dependent pathway starts with mitochondrial pore transition opening, followed by cytochrome C leakage, then interact with apoptosis protease activating factor-1 (Apaf-1) to stimulate activation of caspase-9, then activate caspase-3 [69, 71]. Caspase-3 activation is the common endpoint for intracellular apoptotic process in neurons and glia following cerebral I/R [71, 72]. The activated caspase-3 works as executioner for the apoptosis to cleave kinases, degrade cellular skeletal proteins and DNA-repair enzymes [73]. Target on the caspase-3, the attenuated infarction volume and improved neurological dysfunction was achieved in 2 hours transient MCAO rat model by caspase-3 inhibitor intracerebroventricular injection 1 hour post reperfusion [74]. Therefore, caspase-3 could be a promising target for the intervention of cerebral I/R injury.

2.6.2 Oxidative stress

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Up-regulation of oxidative stress, includes reactive oxygen species (ROS) and reactive nitrogen species (RNS) [75]. Under normal conditions, to maintain homeostasis, once ROS and RNS are produced, which could be scavenged by anti-oxidative system including enzymatic and non-enzymatic systems [76]. Upon the ischemic stroke, the source of oxidative stress originates from mitochondrial inhibition, calcium overload, enzymatic activation, reperfusion, and neuro-inflammatory response [77, 78]. Once the oxidative stress overwhelms the ability of

anti-oxidation, which could directly impair bio-macromolecules (proteins, lipid, and nucleic acid). It is a crucial factor affects the BBB leakage and vasogenic edema, especially post reperfusion [65]. Rao found enhanced endothelial permeability induced by oxidative stress related to regulation of occludin and zonula occludens-1 (ZO-1) [79], which are main components of BBB. Oxidative stress also implicated to the activation of enzymes related to the cerebral I/R damage. For example, increased neuronal nitric oxide synthases (nNOS) and inducible NOS (iNOS) were identified in neurons and vascular endothelium, which are associated with undesirable effects for body [4]. Elevated level of nNOS was detected in the core ischemic brain area at 24 hours of MCAO [80]. Furthermore, mice lacking nNOS [81] and iNOS [82] gene showed less brain damage compared with wild-type counterparts upon MCAO. Another important enzyme activated by oxidative stress is MMP-9 [83]. The underlying mechanism is through activation of oxidation sensitive transcription factors (i.e. NF-κB, activated protein-1) [4]. Collectively, oxidative stress works as a mediator to amplify neuro-inflammation and the brain damage [4, 84-86]. Therefore, oxidative stress is regarded as a crucial treatment target for attenuating the cerebral I/R injury.

Nrf2 is one of the critical anti-oxidative factor, which is a transcription factor to adjust a bank of phase II antioxidant genes synthesis [87, 88]. Under normal condition, Nrf2 combines with regulatory protein, kelch-like erythroid cell-derived protein with CNC homology associated protein 1 (Keap1) in the cytoplasm. Oxidative

stress or electrophilic agents could induce Nrf2 phosphorylation, dissociate Keap-Nrf2 complex and transfer Nrf2 to nucleus. Afterwards, Nrf2 binds to promoter of antioxidant response element (ARE) to mediate related genes transcription. The target genes involve in detoxification of xenobiotics, maintenance of redox potential, and free radicals scavenging [4, 89]. Previous study reported that neuro-protective effect was identified in a calcium overload stroke models through enhancement of Nrf2 activity [90]. Another study found that 24 h post reperfusion, the activity of anti-oxidative enzyme was reduced in the brain of Nrf2-deficient mice, which related to the increased of infarct volume (~10%) in 90 min transient MCAO model [91]. Some reports have proved Nrf2 inducer could reduce infarct size, sensorimotor deficit, brain edema and neuronal cell death in permanent [92] and transient MCAO rats models [93].

2.6.3 Neuro-inflammation

Inflammatory response includes enhanced inflammatory mediators and activation of inflammatory cells [94]. Inflammatory mediators normally consist of cytokines, chemokines, and adhesion molecules etc. As for inflammatory cells, include resident inflammatory cells (microglia), neutrophils, macrophages and T-lymphocytes [94]. The increased inflammatory mediators could trigger more inflammatory cell invasion, for example, TNF-$\alpha$, IL-1$\beta$, and ICAM-1 etc. ICAM-1 as one of crucial factors could induce the firm combination between leukocytes and

endothelial cells to facilitate the leukocytes diapedesis [51, 94, 95]. In addition, elevating invasion of inflammatory cells causes further release of free radicals contribute to the progression of cerebral I/R. Moreover, increased leukocytes and oxidative stress as sources of abundant inflammatory mediators [96], could trigger the over-expression of MMP-9. MMPs are $Ca^{2+}$ and $Zn^{2+}$ dependent endopeptidases with three domains: a hemopexin-like C-terminus, a catalytic domain and an N-terminal propeptide [94, 95, 97]. As one of the downstream factors of inflammation [98] and free radicals [83, 99], MMPs exert main efforts to cause hemorrhagic transformation and brain edema through directly degrading extracellular matrix and TJ proteins [61], which are two main components of BBB. In addition, MMPs are able to activate other pro-MMPs, induce pro-inflammatory cytokines and facilitate leukocytes infiltration [100, 101]. With the synergistic effects of oxidative stress and inflammation, the brain cell death is exacerbated upon the ischemic stroke [102]. Accumulating evidences have shown elevated MMP-9 [27] was observed both in *in vitro* and *in vivo* experiments upon I/R stimuli [103-109], and even in patient [110]. Elevated MMP-9 related to later stage of BBB opening post-reperfusion [61]. Inhibitor or gene knockout of MMP-9 can attenuate leukocytes adhesion [26] and restore the damaged BBB function [27, 106, 107, 111], which reduced risk of hemorrhagic transformation and brain edema.

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NF-κB plays ubiquitous role in inflammation, immune responses, cell division and apoptosis [112]. NF-κB is a multiprotein complex that resides in many cells types in the neuro-system, and this factor might be activated through a number of stimuli involved in cerebral I/R, including glutamate, pro-inflammatory cytokines, and the reactive oxygen species. Under physiological conditions, NF-κB is expressed in the cytoplasm combined with the inhibitor protein, IκB. Upon stimulation, IκB is phosphorylated through IκB kinase (IKK), and the p50 and p65 (RelA) subunits of NF-κB are subsequently translocated to the nucleus, where these molecules could bind to target DNA and induce the synthesis of mRNA [113, 114] associated with inflammation, oxidative stress and apoptotic processes in cerebral I/R, such as, TNF-α, IL-1β, ICAM-1, MMP-9, iNOS, Bcl-2 and caspase-3 production [44, 112]. Therefore, NF-κB works as converge point regulates pathological cascade of stroke, which might be regarded as a critical therapeutic target in neurodegenerative diseases.

Currently the thrombolytic treatment is the only approved therapy for acute ischemic stroke by Food and Drugs Administration (FDA) [115], which means restoration of blood circulation to the ischemic brain area [115]. However, the utilization of blood restoration is confined by its narrow treatment window (4.5 hours post stroke onset) [115], in addition, the therapeutic effect was partially counteracted by I/R injury through anabatic excessive production of oxidative stress, inflammatory responses and brain cell death [8], which could enhance adverse risky of

hemorrhagic transformation, and cerebral edema, especially delayed manipulation of thrombolysis [43].

To decrease I/R injury and optimize the recanalization therapeutic effect, accumulating studies have focused on the biomarkers of stroke pathophysiological pathways and explored ample neuro-protective agents with successful intervention effects in *in vitro* and *in vivo* experiments; however, with no exception, these treatments have failed in randomized clinical trials [116]. Therefore, recent studies have been focused on daily dietary phytoalexin, particularly multi-target agents, for the treatment of stroke.

Curcumin is a phenolic pigment extracted from the rhizome of *Curcuma Longa* Linn [117]. Three main components normally included into the commercial curcuminoid powder, curcumin, bisdemethoxycurcumin (BDMC) and demethoxycurcumin (DMC). Curcumin, identified as diferuloylmethane ([1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione]) (Figure 2.1), is the main ingredient among curcuminoids in turmeric [8]. The main structure of curcumin includes phenolic molecules, which could provide H-atom to directly scavenge free radicals [118]. The most important metabolism steps of curcumin by oral taken involved in reacting with endogenous enzyme in gut and phase I and II enzymes in liver, result in the conjugation, includes glucuronide and sulfate [119]. That will influence its bioactivity. Curcumin is a multi-functional substance with a variety of

cellular effects on different diseases [120]. The protective effect mainly attributed to its anti-inflammation and anti- oxidation effects [118]. Several studies have reported that curcumin has strong neuro-protective effects on neuro-injury ailments [120], with extremely safety, even administration with high dose [121].

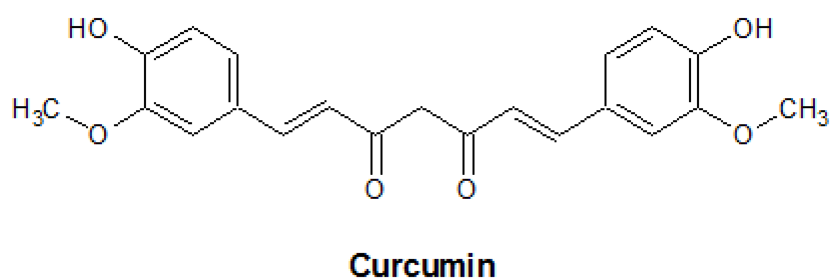


*Figure 2. 1 The molecular structure of Curcumin.*

The neuro-protective effects of curcumin against cerebral I/R damage are primarily achieved through restoring BBB disruption [122], decreasing cerebral infarction volume [123], ameliorating brain edema [124] and improving neurological motor function deficit [125] via anti-oxidant, anti-inflammation and anti-apoptotic properties [126, 127]. The inhibitory effects of curcumin on NF-κB activation and subsequent ICAM-1 gene expression have been demonstrated in TNF-α-stimulated HBMVECs [40].

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# CHAPTER 3

## METHODOLOGY

The process of our research was that stroke model induction followed by intervention administration at 30 min later, the neurological deficit score was detected at another 30 min later, then followed by reperfusion operation, finally collect all the other parameters at 24 hours after reperfusion as shown in Figure 3.1

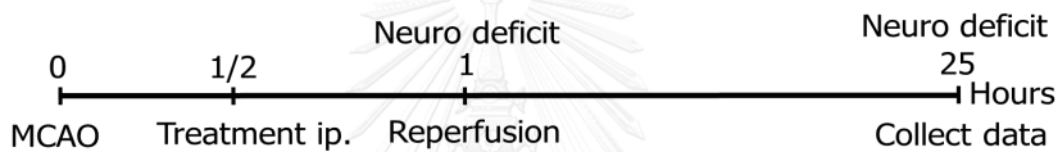


*Figure 3. 1 Schematic diagram shown the experimental procedures*

### 3.1 Animal preparation

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74 male Wistar rats (10 weeks age, body weight 250-300 g) were used from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Nakornpathom, Thailand. All experimental procedures of this study were approved by Ethics Committee, Faculty of Medicine, Chulalongkorn University, and administered following the guideline of experimental animals announced by the National Research Council of Thailand (1999). The animals were housed in animal center with 12:12 hour light-dark cycle for at least 1 week before experiment. All rats could access to normal chow and tap water ad libitum.

3.2 Pilot studies of 1 h and 2 h MCAO models

For pilot study, we tried 2 or 1 hour occlusion followed by 24 hours reperfusion, respectively. There was no significant difference in neurological deficit scores between 2 h (3.833 ± 0.3218) and 1 h (3.207 ± 0.2450, $P$=0.1571) occlusion groups as shown in Figure 3.2.B. However, the infarction volume was significantly higher in 2 hours MCAO group than that in 1 hour MCAO group (50.41 ± 5.601 *vs*. 32.96 ±1.871, *$P$=0.0120) in Figure 3.2.A. In addition, the number of dead rats was much more in 2 hours group (4/12) than that of 1 hour group (5/29). 17% death rate was found in 1 hour transient MCAO model of our study, which was quite similar with the result reported by Odilo [128], in his study, 10-20% of the death rate was found, which was depended on the occlusion duration for MCAO model. In addition, for 2 hours occlusion group, the rat couldn’t bear the operation for BBB leakage checking on the second days. Therefore, 1 hour occlusion followed by 24 hours reperfusion was adopted for present study.

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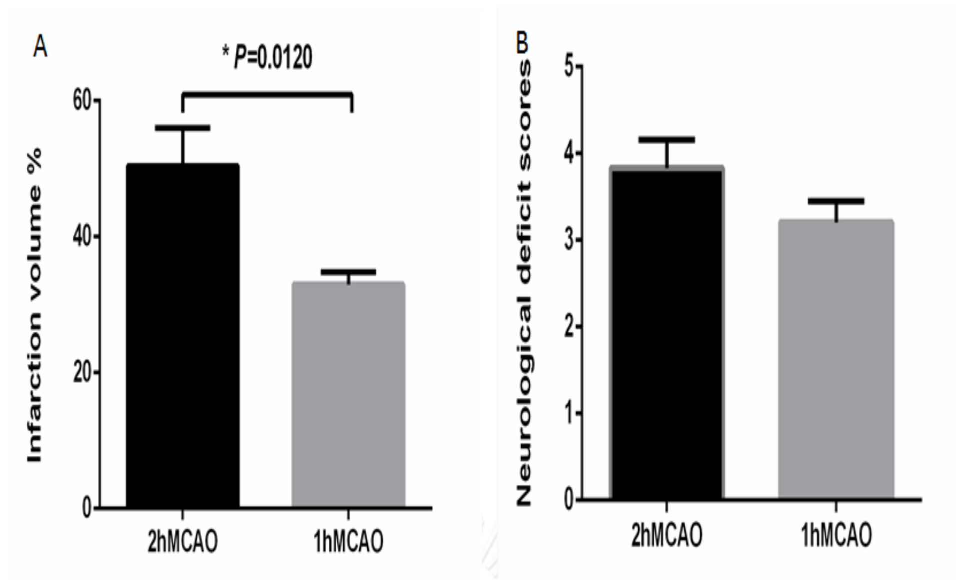


*Figure 3. 2 The difference in neurological deficit and infarction volume between 1 and 2 hours MCAO model. The significant higher infarction volume was observed in 2 hours MCAO than 1 hour MCAO group (*P=0.0120, n=7 rats/group) (A). No significant difference was found in neurological deficit scores between 1 and 2 hours MCAO model (B);*

3.3 Classification of animal

All rats were randomly distributed into four groups. Because all parameters could not be acquired from each rat, therefore, the sub-groups of animals were set at minimum requirement for statistical analysis as shown in the experimental diagram (Figure 3.3), respectively.

Group 1: SHAM operation group treated with 1ml corn oil, i.p (SHAM+CORN, n=9)

Group 2: SHAM operation group treated with 300mg/kg BW curcumin, i.p (SHAM+CUR, n=9)

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Group 3: MCAO group treated with 1.0 ml corn oil, i.p. (MCAO+ CORN, n=29)

Group 4: MCAO group treated with 300 mg/kg BW curcumin, i.p (MCAO+CUR, n=27)

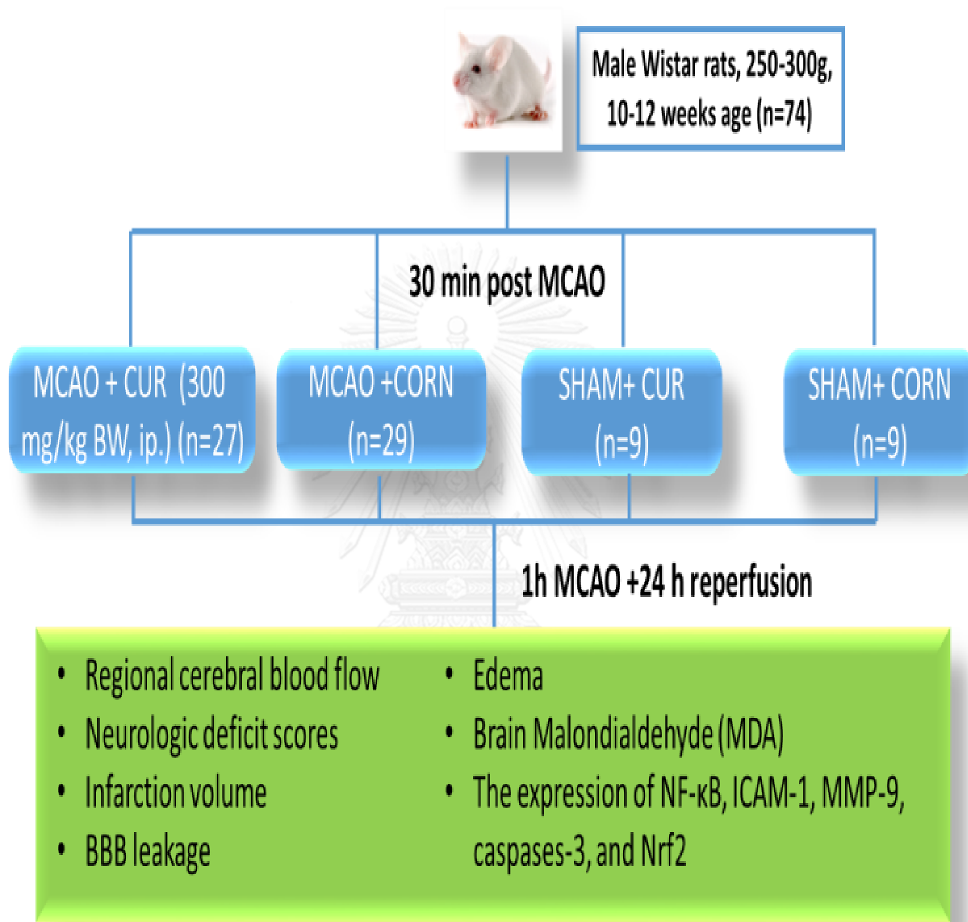


*Figure 3. 3 Four groups of rats were subjected to the experimental procedures for data collections of each parameter.*

3.4 Cerebral I/R injury induced by MCAO Model with monofilament

The method of MCAO model was performed according to the Wang's research [129] as following:

Preparation of MCAO

1. Operation instruments (as shown in Figure 3.4)

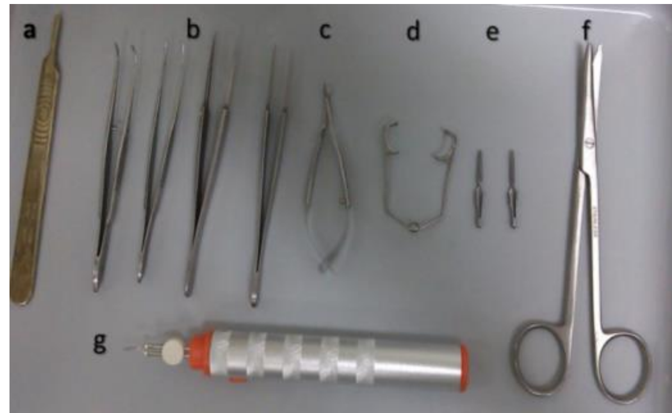


*Figure 3. 4 Microsurgical instruments were demonstrated. (a). Scalpel (b). Microdissecting forceps (c). Microdissecting scissors (d). Microdissection retractors (e). Microvessel clamps (f). Operation scissors (g). Coagulation unit*

2. Occlusion tool

4-0 Nylon monofilament suture (Cat.403956#) was commercial available from Doccol company, the tip of it was coated with silicon rubber, and white mark was made with correction pen at 20 mm long from its tip. The diameter of coating area was 0.36+/- 0.02 mm, coating length is 5-6mm.

Experimental protocol

1. The rat was anaesthetized with inhalational anesthetics machine (induction with 5 % of isoflurane and maintenance with 2-3 % isoflurane; SurgiVet, Giss marketing Co., LTD, Thailand).

2. The homoeothermic pad (Comella', Spain) was used to keep the rat body temperature at 37°C during the whole operation.
3. Placed the rat on supine position, and sterilized surgical field with 70% ethyl alcohol.
4. A midline incision was prepared on the neck, then carefully freed the left common carotid artery (CCA) without damaging the vagal nerve.
5. The external carotid artery (ECA) was freed and a knot was tied on the far-end from the heart with No. 6.0/7.0 suture.
6. The internal carotid artery (ICA) was separated and the blood flow of CCA and ICA were blocked with micro vessel clamps.
7. A small incision was made on the ECA away from bifurcation of CCA. The monofilament with silicon-coated tip was advanced into the ECA until it reach to ICA clamps.
8. A knock was made to tighten the monofilament outside of ECA, then the clamp of ICA was removed.
9. Inserted the monofilament into ICA around 16-20 mm long (Figure 3.5 A) until slight resistance was felt (if shorter than ∼ 10 mm, means mis-insertion into pterygopalatine artery), which means blockage of the origin of MCA (Figure 3.5 B).
10. Fastened the knock outside of ECA stump to fix the monofilament.
11. Removed the clamp of CCA and closed the wound layer by layer.

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12. The rat was allowed to wake up in a new clean cage for 30 min before reperfusion.
13. Under isoflurane anesthesia, reopened the wound and blocked the blood flow of CCA with micro clamp.
14. Removed the knock of ECA stump, and gently withdrawn the monofilament from the blood vessel to perform reperfusion.
15. Closed the ECA with suture and closed the wound again.
16. The rat was allowed to wake up for another 24 hours in an individual clean cage.

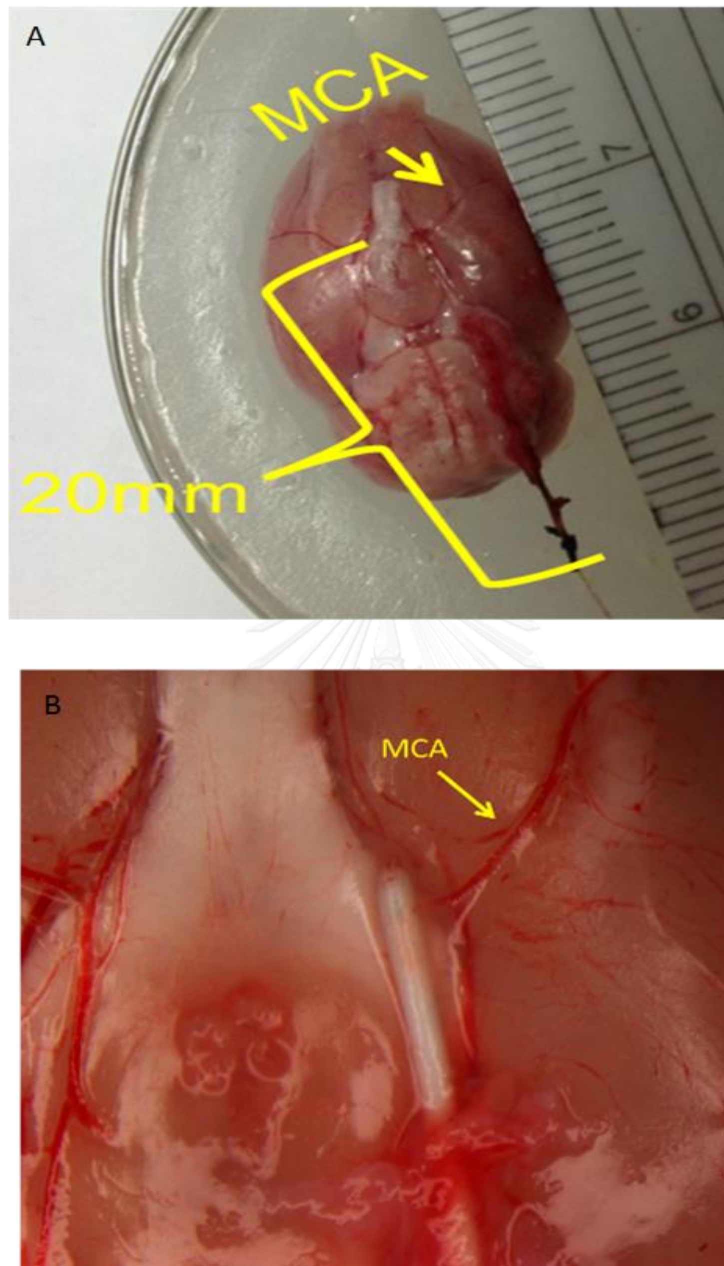



*Figure 3. 5 The post mortem examination of stroke model. The length of inserted monofilament in the blood vessel was about 20 mm (A); the MCA was occluded by silicon coated monofilament (white color) (B).*

SHAM Operation

The rat of SHAM group was subjected to the same operation steps as MCAO group, but except the MCA occlusion.

3.5 Drug administration

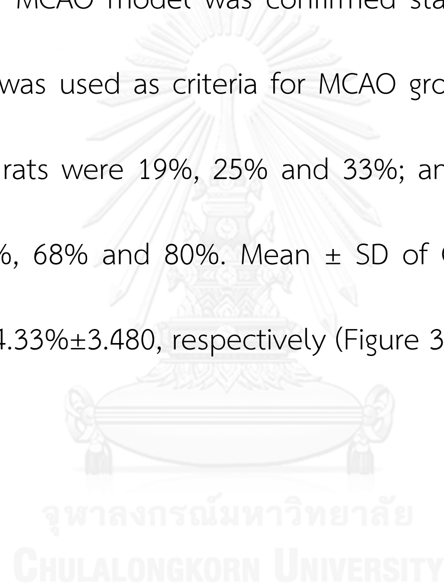
The vehicle or curcumin was administered according to classification of experimental rat. The method was modified from Triyagarajan's research [5]. Curcumin (purity≥90%, Cayman chemical company, Cat 81025, USA) was solubilized in 1.0 ml corn oil and injected intraperitoneally with dose of 300 mg/kg body weight at 30 min after MCAO.

3.6 MCAO model confirmation

3.6.1 Regional cerebral blood flow (CBF) detection

This experiment was performed according to the previous studies [8, 130, 131]. Laser-Doppler perfusion monitor (LDF; PeriFlux System 5000, Perimed AB, Sweden) was used to check the MCAO model induction. The skull surface of distal middle cerebral artery supply territory (4 mm lateral, 2.0 posterior to the bregma) was thinned by a dental drill (World Precision Instrument Inc., USA). Then the probe (PeriFlux System 5000, Perimed AB, Sweden) of LDF was perpendicularly fixed ∼1 mm above with dental cements, and avoid the big vessel underneath (see Figure 3.6 A). The result of CBF was continuously monitored during the whole operation procedure (start from 20 min pre-occlusion to 20 min post reperfusion). Total 3 rats

in MCAO group were chosen to perform this experiment. The criteria of MCAO model inclusion was that, CBF should immediately decline lower than 35% of the pre-occlusion level (set at 100%) right after MCAO, and maintain at low level until reperfusion; the level of CBF should recovery higher than 60% of pre-ischemia value within 30 min after reperfusion [129]. Owing to the extra damage could be added to the rat, which could make rat hard to survive for other parameters detection. Once the operation skill of MCAO model was confirmed stable by this test, neurological dysfunction monitor was used as criteria for MCAO group inclusion. After occlusion, the CBF results of 3 rats were 19%, 25% and 33%; and the results of 20-min after reperfusion were 75%, 68% and 80%. Mean ± SD of CBF at two time points were 25.67%±4.055, and 74.33%±3.480, respectively (Figure 3.6 B).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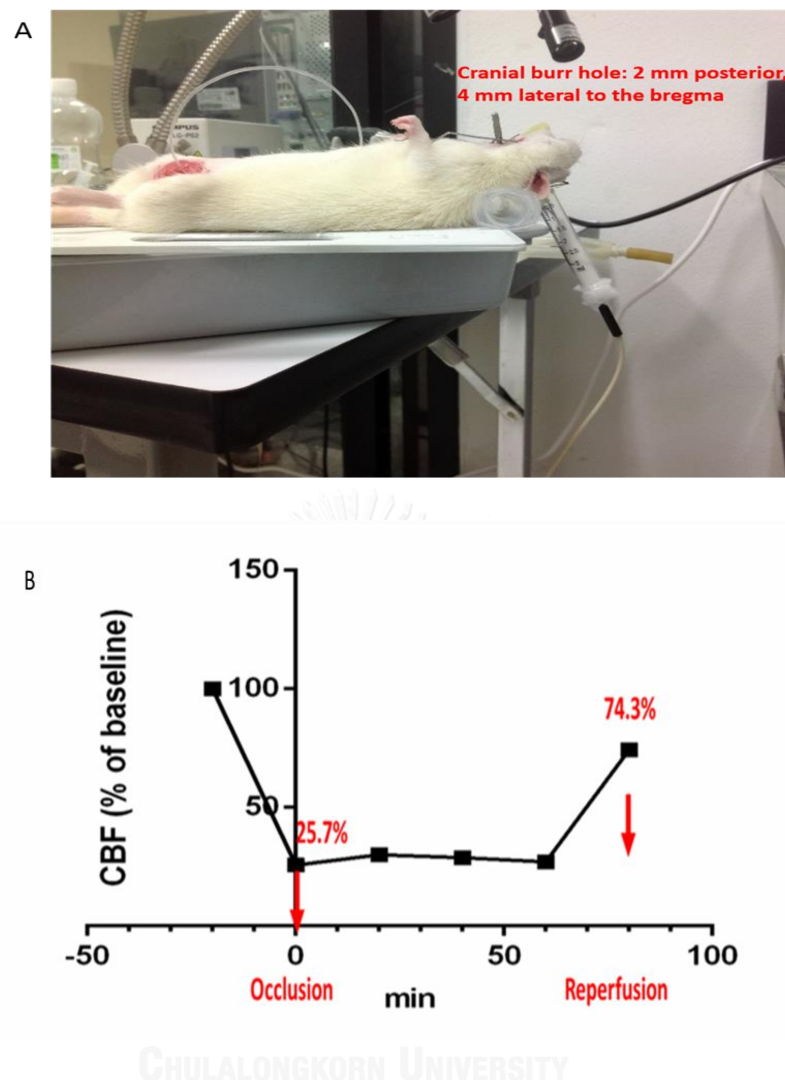


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*Figure 3. 6 The CBF detection by Laser Doppler flowmetry (LDF). CBF test through LDF, the probe was fixed on the MCA territory (A). To monitor the regional blood perfusion, the cranium was thinned by dental drill at the position of 4 mm lateral, 2 mm posterior to the bregma, then a Laser-Doppler flowmetry probe was fixed on MCA territory. The CBF data was continuously recorded from 20 min before occlusion to 20 min after reperfusion, the final result was expressed as percentage of the mean of 20-min pre-occlusion value (B).*

3.6.2 Neurological deficit scores test

This experiment was performed two times according to Shih's study [93], the first time was conducted right before reperfusion to check the MCAO model induction. The second time was performed before sacrificing rat. With scoring scale system according to Ansari's study [132]. Animals were scored in five categories: 0 correspond to no observable deficits, 1 correspond to failure to extend contralateral forepaw (mild focal neurologic deficit), 2 correspond to circling in a direction contralateral to infarct side (moderate focal neurologic deficit), 3 correspond to falling in a direction contralateral to infarct side (severe focal neurologic deficit), 4 correspond to depressed level of consciousness without spontaneous movement, 5 correspond to dead. The inclusion criteria for MCAO rat group was that the neurological score should be higher than 0 and lower than 5. For MCAO group, including corn oil and curcumin treated group, the neurological deficit scores were 3.00 ± 0.1646 and 3.00 ± 0.1510, respectively (Figure 3.7). There was no significant difference between each other, but the scores of both groups were higher than the score of SHAM group (0).

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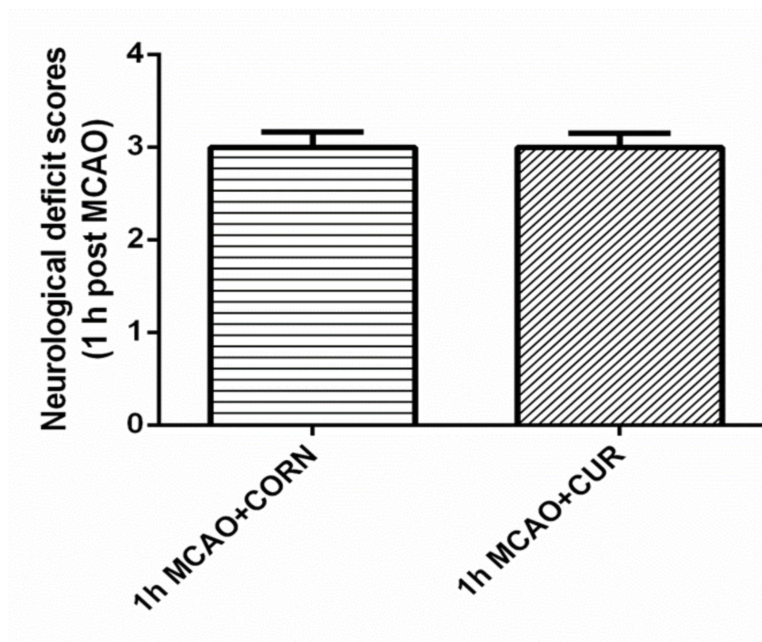


*Figure 3. 7 Before reperfusion, the scores for SHAM groups are 0. There were no significant difference between MCAO+CORN and MCAO+CUR groups for neurological deficit scores.*

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3.7 Assessment of the physiological parameters

To maintain the stable physiological condition for each rat, and decrease the influence of variable physiological status to the cerebral microcirculation. Physiological parameters were measured two times, before preparing the cranial-window for BBB leakage detection, and after BBB leakage detection. First, mechanical respiration was conducted by polygraph system (Nihon Kohden, Japan). Then the cannulation on the left CCA was performed to determine blood gases (pH, PaCO2,

and PaO2). Later blood pressure was measured through left CCA cannulation by blood pressure transducer (i-STAT® analyzer, Abbott Company, USA).

3.8 Blood-brain barrier leakage

The measurement of BBB disruption was modified according to methods of preliminary researches [133-137]. 5 rats/group were used for this study.

3.8.1 Cranial-window preparation

After 24 hours of MCAO, the tracheotomy of rat was performed for mechanical inhalation with ventilator (Kent Scientific, USA) under anesthesia with pentobarbital sodium (60 mg/kg body weight, i.p.). The body temperature was maintain at 37 Celsius with homoeothermic pad (Comella', Spain). Right jugular vein was canulated for injection of Fluorescence isothiocyanate-labeled dextran-250 (FITC-dextran-250, 50 mg/ml, Sigma Chemical Co., USA) and supplement of anesthesia. Then left CCA was canulated for blood gas and pressure measurement. The head of rat was placed in custom-made stereotactic frame. The craniotomy was performed over the left MCA territory (coordinate: 2 mm lateral, 2 mm posterior to the bregma; 2×2-mm burr hole) with a dental drill. The dura was pierced with a 30-gauge needle and cut with ophthalmic scissor. After carefully retracted the dura and exposed the surface of the brain, a lab-made chamber was attached over the burr hole. Then the cranial window was suffused with artificial cerebral spinal fluid (aCSF)

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(2 ml/min) [137] that continuously bubbled with 95% nitrogen and 5% carbon dioxide [134]. Temperature of the suffusate was maintained at 37±1$^{o}$C (Figure 3.8).

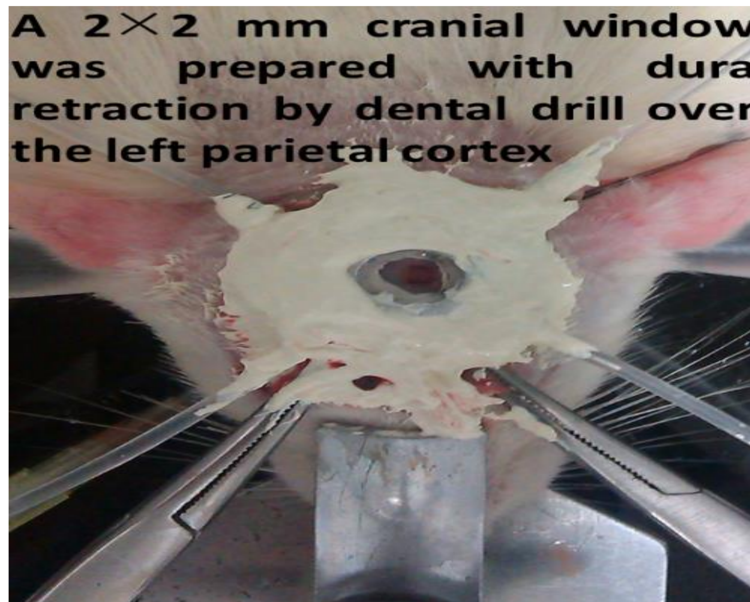


*Figure 3. 8 The cranial window was prepared for BBB leakage detection*

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3.8.2 BBB disruption detection

To visualize BBB disruption, 0.2 ml 5% FITC-dextran-250 was administered intravenously into jugular vein [138]. At 0 and 10 minutes after FITC injection, the fluorescein images were captured by the confocal laser scanning microscope (Nikon, Japan) with 488nm and 512nm blue light excitation and emission wave length. Capillary permeability was represented by relative fluorescence units measured by the flux of FITC-Dx-250 across the capillary wall (Figure 3.9). Image-Pro plus 6.0

software was used to analyze images (Figure 3.10), then the percent change of extravasation of FITC was calculated by the equation of: [135]

% Leakage = [(Iout / Iin) at 10 min - (Iout/Iin) at 0 min] / [(Iout/Iin) at 0 min] ×% [135]

Iout = Fluorescent Intensity outside capillary

Iin = Fluorescent Intensity inside capillary

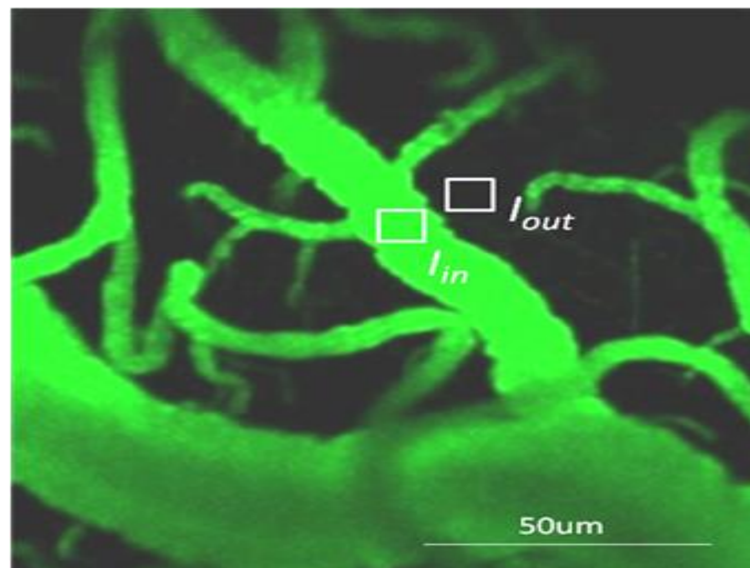


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*Figure 3. 9 The region of interest for BBB leakage detection*

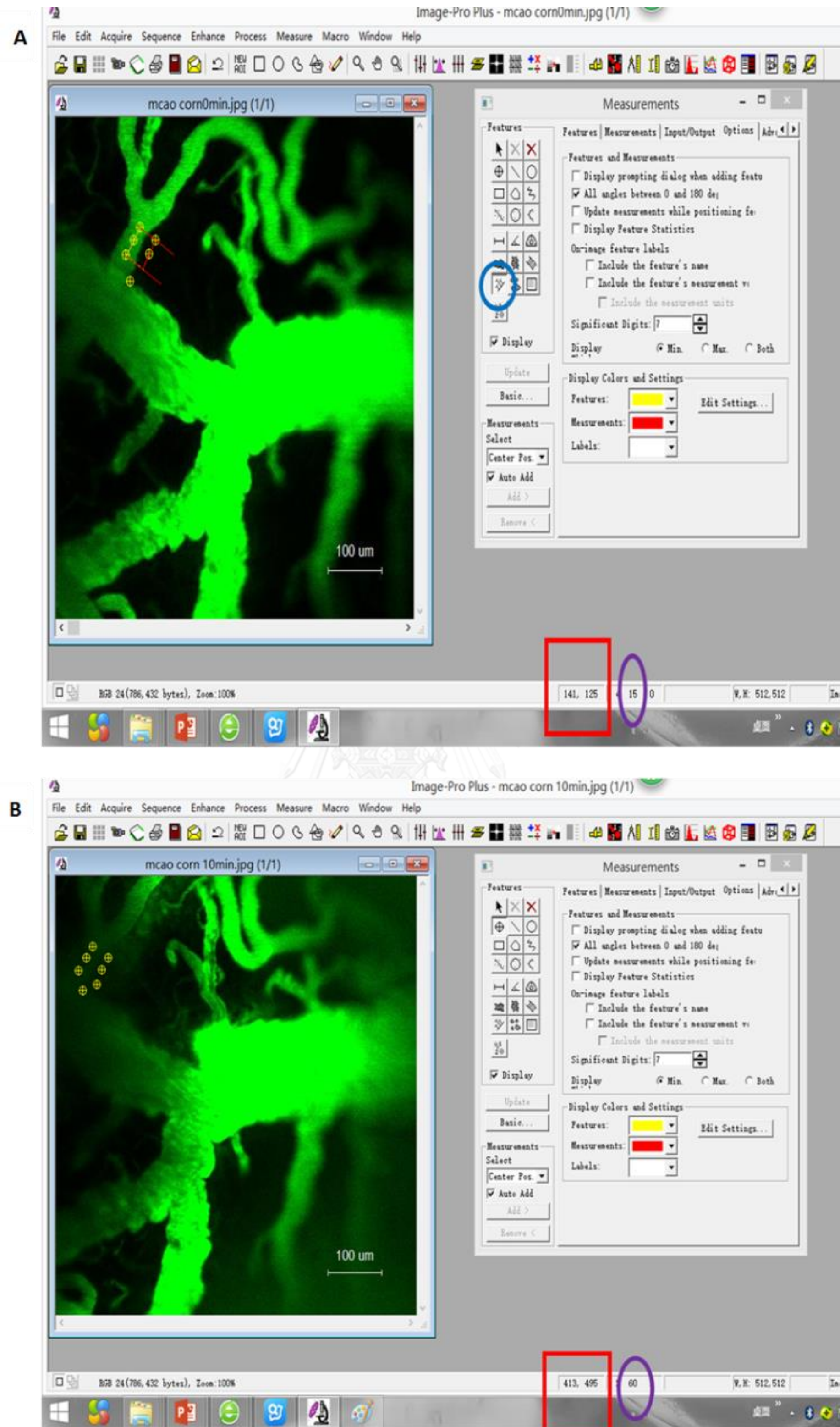


*Figure 3. 10 The BBB leakage detected by Image-Pro plus 6.0. After calibration, at 0 min post FITC injection, the 20- 40 μm diameter of post capillary venule was*

*focused, be sure no obvious capillary network on the background, the bifurcation point of blood vessel and 3 points (yellow color) in the midline of intra-blood vessel were fixed; by using one function of measurement (blue color) to fix parallel 3 points outside of blood vessel, and read the corresponding coordinate in red square frame and the pixel in purple circle, the average pixel was calculated for inside/outside of capillary Iin/Iout at 0 min (A). At 10 min post FITC injection, the corresponding 6 points (3 inside, 3 outside of blood vessel) were fixed by using coordinate of reference point in Figure A (bifurcation point); the pixels was read from purple on the bottom. The average pixel of each point was calculated for Iin/Iout at 10 min (B) as well.*

3.9 Brain water content measurement

To detect the edema, brain water content was measured by method described by Yang [92]. 3 rats/group for SHAM group, 5 rats/group for MCAO group were utilized for this test. Under anesthesia with pentobarbital sodium (100 mg/kg body weight, i.p.), rats were decapitated, the left and right hemispheres were divided quickly, and the wet weight of each side was weighted by electronic balance. Then the brain tissue was dried in the oven at 70 °C for 48 hours, the dry weight of each hemisphere was obtained, with the following formula, the brain water content was calculated:

water content (%) = (wet weight — dry weight)/ wet weight ×100% [92].

3.10 Analysis of infarction volume

To check the infarction volume, 7 rats/MCAO group were used. The method was modified based on Durukan's research [139]. After sacrificed and decapitated the rat, seven coronal slices (2-mm thick) was cut with the brain-cutting matrix. Brain slices were bathed in the 2% TTC solution at 37°C for 15 min, then fixed in the 10% formalin for 24 hours. Then mounted slices on black tissue and photographed with a digital camera for further planimetric lesion size analyses with Image-Pro plus 6.0 software (Figure 3.11). The total infarct volume of whole brain was obtained by adding up with infarction volume of each slice. To adjust the influence from edema, the correction of infarct volume on each slice was calculated by equation:

Infarction volume %=

$$\frac{[(\text{contralateral hemisphere area})-(\text{ipsilateral hemisphere non-injury areas})]\text{x}2\text{mm}\text{x}100}{\text{Contralateral hemisphere area}}$$

[139]

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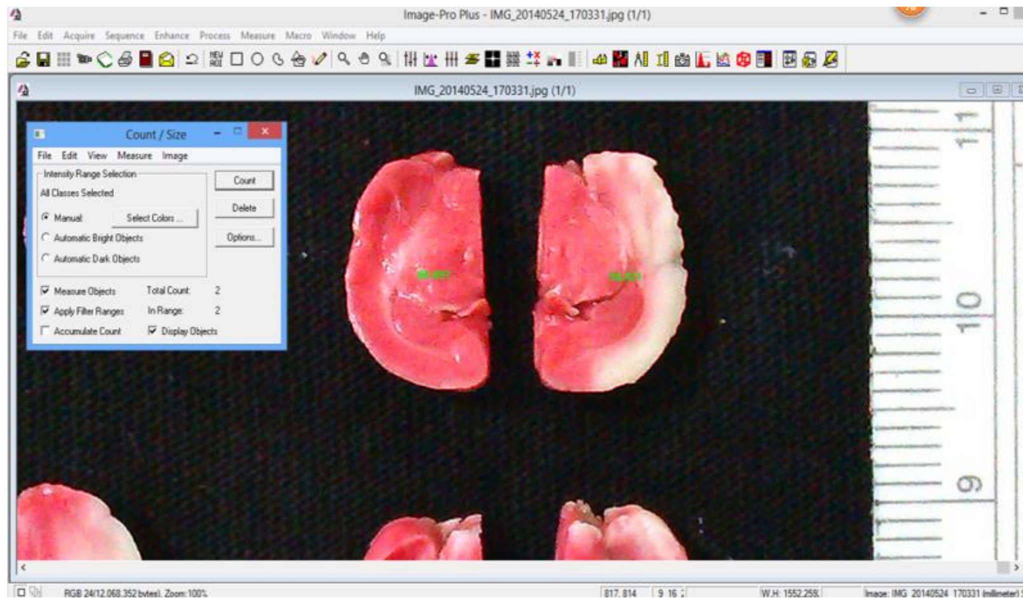


*Figure 3. 11 Infarction size measured by Image-Pro plus 6.0. After calibration, by using function of Annotate to draw the infarction region (white color) and non-infarction region (red color), then by using count/size function to get size of each region.*

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3.11 Tissue preparation and homogenate

1. Plugged a catheter into the left ventricle of rat heart for perfusion under Nembutal anesthesia (100 mg/ body weight).

2. Perfused the rat with 200 ml ice cold heparinized (25U/ml) phosphate buffer saline (PBS, 0.01M PH 7.4).

3. Removed the whole brain quickly without cerebellum and brain stem after decapitated the rat.

4. Isolated around 200 mg of brain tissue from injury and non-injury areas of both hemispheres.
5. Transferred the freed brain sample into a low temperature (4 °C freezer) treated mortar.
6. Added 10 ml liquid nitrogen to the mortar and soaked sample for several seconds.
7. Pulverized the sample for 1-2 min with Pestle.
8. Transferred the sample to a micro tube containing ice cold lysis buffer (1000 μl of lysis solution: 100 mg sample), the buffer was consisted of RIPA buffer (Cell Signaling, Beverly, MA), and protease inhibitor (10 μl protease inhibitor: 1000 μl lysis buffer, Sigma Co., USA).
9. Vortexes for 15 sec; rested on ice surface for 30 sec; repeated the process until 30 min.
10. Centrifuged sample at 12,875 g for 10 min at 4 °C.
11. Pipetted 200 μl supernatant into micro tube at 100 μl each time.
12. Stored the supernatant at -80 °C until MDA and BCA testing.

3.12 Estimation of oxidative stress markers

To measure oxidative stress in the injury brain tissue, the same rats of BBB leakage detection were used. Malondialdehyde (MDA) was estimated at the endpoint

of experiment. MDA is a biomarker for lipid peroxidation. After perfused and sacrificed the rat under anesthesia, the brain was removed and separated into two hemispheres, the level of MDA in injury and non-injury hemispheres was tested by MDA TBARs assay kit (Cayman cor. USA) according to the manufacturer's introduction. This protocol was modified based on study of Wang et al. [140].

3.13 Immunohistochemistry assay

To detect the distribution and expression of MMP-9, ICAM-1, Nrf2, caspase-3 and NF-**K**B in the brain, the immunohistochemistry was used [141]. 7 rats per group were utilized for this test. Brain tissue of MCA territory was obtained (3mm - 6mm posterior to bregma) [130] and fixed in 4% paraformaldehyde at room temperature for 24 hours, then embedded in paraffin box; sequentially sectioned the brain tissue into slices with 2 um thickness. The sequence of slices was recorded from frontal copular part to occipital part, every 5 slices were repeatedly incubated with five different primary antibodies. Totally 2 slices for each protein detection. After de-paraffinizition and antigen retrieval process (DaKo, Denmark), the slices were successively incubated with 3% $H_2O_2$ and diluted antibody (DaKo, Denmark). Then incubated with anti-mouse ICAM-1 (Cat: 554967, 1:50 dilution, BD Pharmingen, USA) and MMP-9 (Cat: ab58803, 1:700 dilution, Abacam, UK), anti-rabbit caspase-3 (Cat: 9662s, 1:700 dilution, Cell Signaling, USA), anti-rabbit Nrf2 (Cat: ab31163, 1:100 dilution, Abcam, UK) and NF-**K**B-p65 (Cat: 8242s, 1:800 dilution, Cell Signaling, USA) at

4 °C overnight. The immunoreactivities were detected by DAB detection kit (DaKo, Glostrup, Denmark). Nuclei were stained with hematoxylin. The sections were photographed at 400× magnification in four interest regions (two for penumbra, two for core areas) (appendix A) of the MCA territory area via a light microscope (Nikon eclipse TS100, Japan). 4 pictures per region were captured; the immunoreactive cells number (nucleus and cytoplasm positive staining) was separately counted by manual for Nrf2, NF-κB-p65 and caspase-3. The result was represented by number of positive cells/total area. Image-Pro plus 6.0 was used for analyzing positive staining of ICAM-1 and MMP-9. Which is modified from Jiang's study [142]. The optical density was calibrated, then color-specific threshold was set as: hue, 0–29; saturation, 0–255; intensity, 0–110 (ICAM-1); hue, 0–29; saturation, 0–255; intensity, 0–100 (MMP-9) (Figure 3.10). The result was represented by integrated optical density (IOD)/total area. Results of non-injury side was regarded as SHAM operation control.

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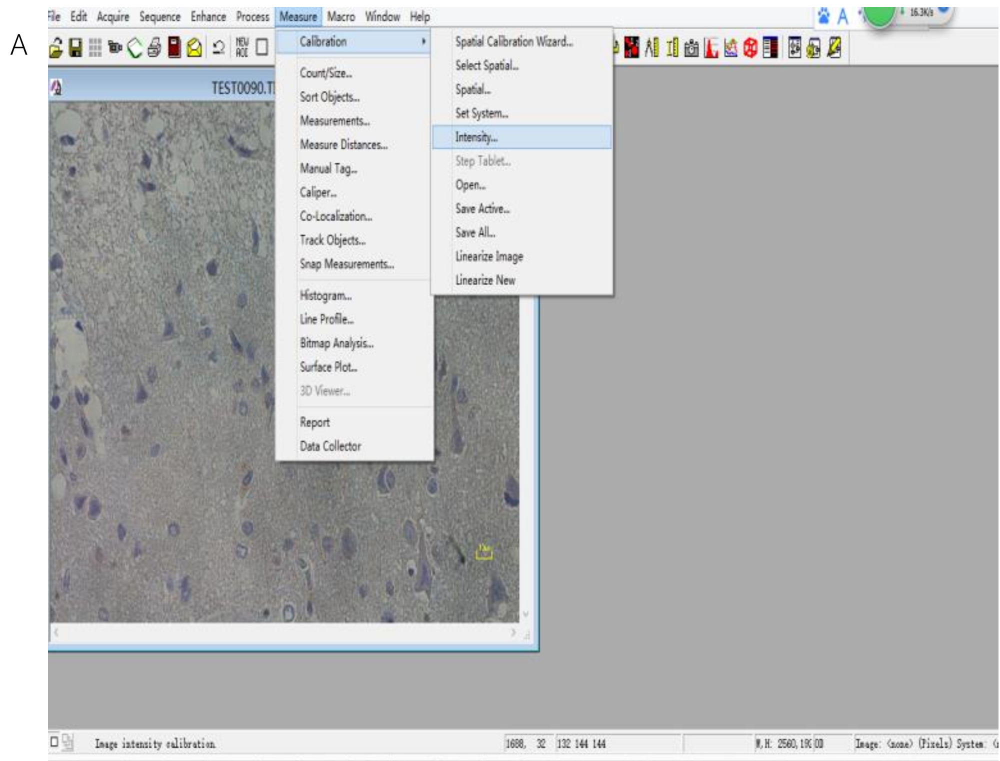
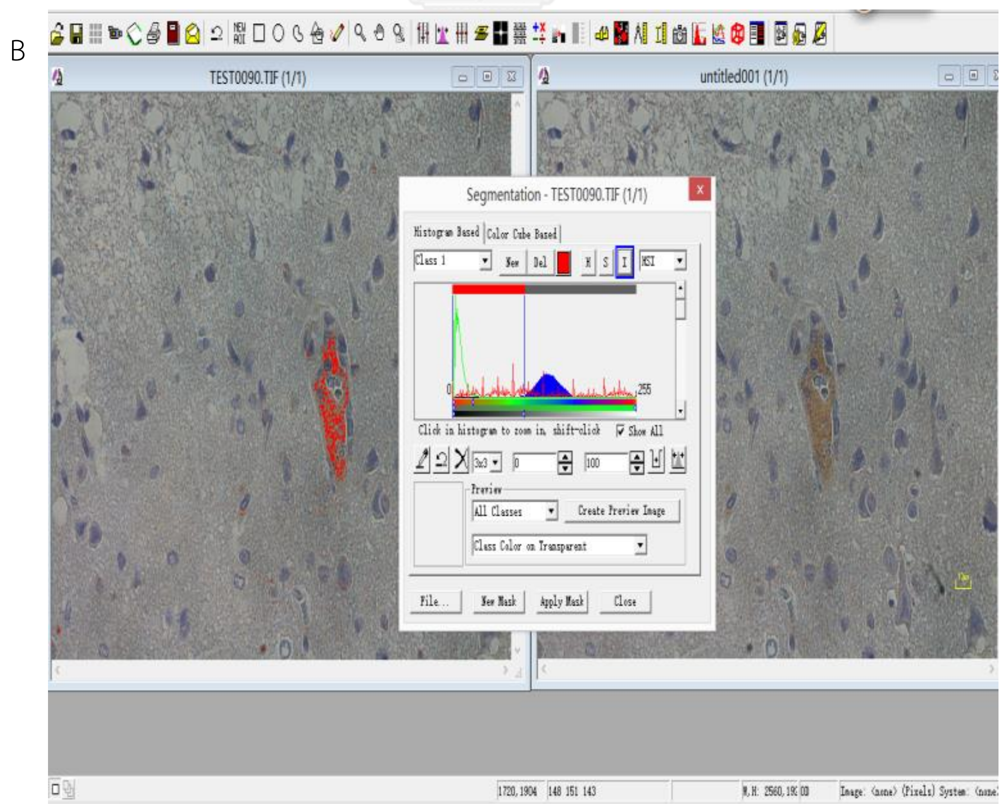
A




B



C

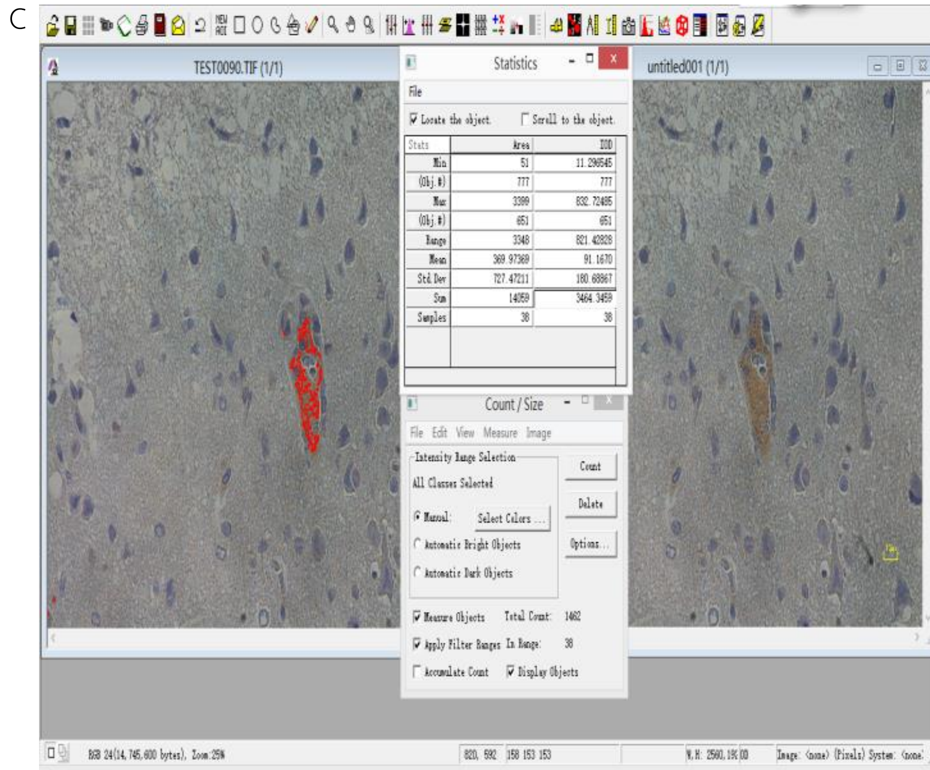


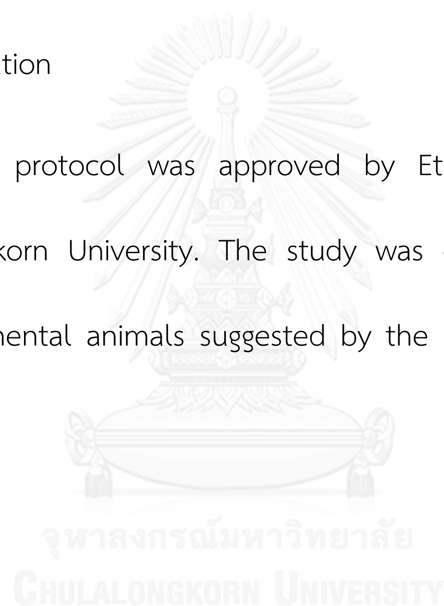

*Figure 3. 12 By using Image-Pro plus 6.0 to analyze the MMP-9 and ICAM-1 with integrated optical density method. First calibrate the intensity (A); by using histogram to focus the brown color area (positive staining) (B); measure the IOD of interest (C).*

### 3.14 Statistical analysis

All results were expressed as the means ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was made to examine the difference of each parameter, followed by the Post Hoc test. Neurologic deficit scores was analyzed by Mann-Whitney U test [92]. Differences was considered significant at $P < 0.05$.

### 3.15 Ethical consideration

The study protocol was approved by Ethics Committee, Faculty of Medicine, Chulalongkorn University. The study was conducted according to the guideline for experimental animals suggested by the National Research Council of Thailand (1999).



# CHAPTER 4

## RESULTS

### 4.1 Neurological deficit scores at 24 hours post reperfusion

After 1 hour occlusion followed by 24 hours reperfusion (25h MCAO), the neurological deficit scores were detected again to check the effect of cerebral I/R on the neurological function. This time the rat with score 5 was not excluded from group. Our results illustrated that the scores was 3.207 ± 0.2450 in MCAO+CORN group, it was significantly higher than SHAM group (0). However, the result was significantly lower in curcumim treated MCAO group (2.259 ± 0.2647) than corn oil treated group (*$P<0.05$) as shown in Figure 4.1.

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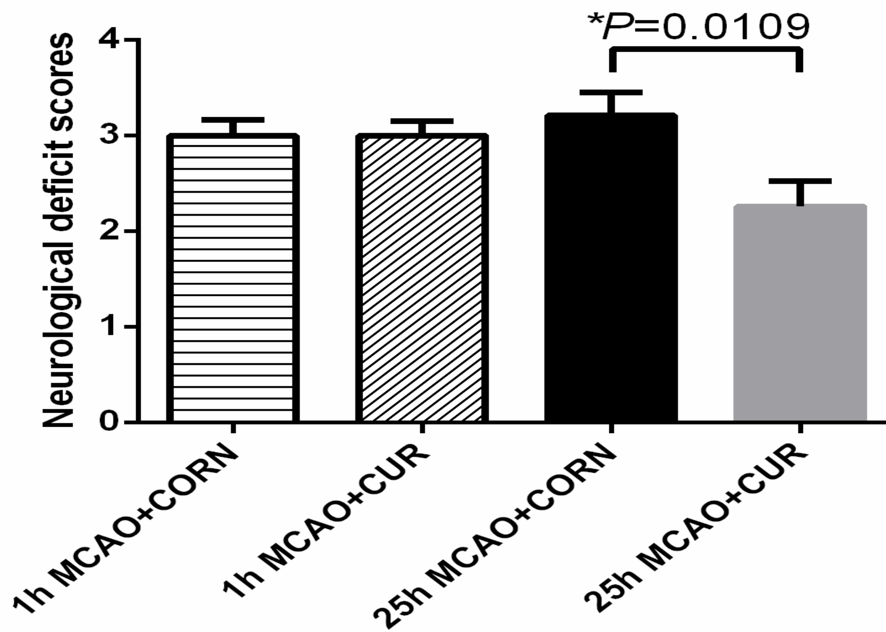



*Figure 4. 1 The neurological deficit scores. This experiment was performed two times, the first time was conducted for confirming the stroke model. For 1h MCAO groups, the results were similar between corn oil and curcumin treated group. The second time to conduct this experiment was to check the effect of curcumin on neurological dysfunction at 24 hour post reperfusion. The results shown significantly lower scores in curcumin treated group than corn oil treated group.*

4.2 Infarction volume

Infarction volume was detected by 2% TTC staining. Figure 4.2 A showed that the infarction volume (white area) of MCAO+CORN group appeared much larger than MCAO+CUR group; there was no obvious infarction size in SHAM group. Figure 4.2 B showed that MCAO+CORN group (32.96% ± 1.871%), the infarction size was significantly higher than SHAM group (0, #*P* < 0.0001), which was significantly reduced in curcumin treated MCAO group (26.91 ± 1.563, **P*< 0.01).

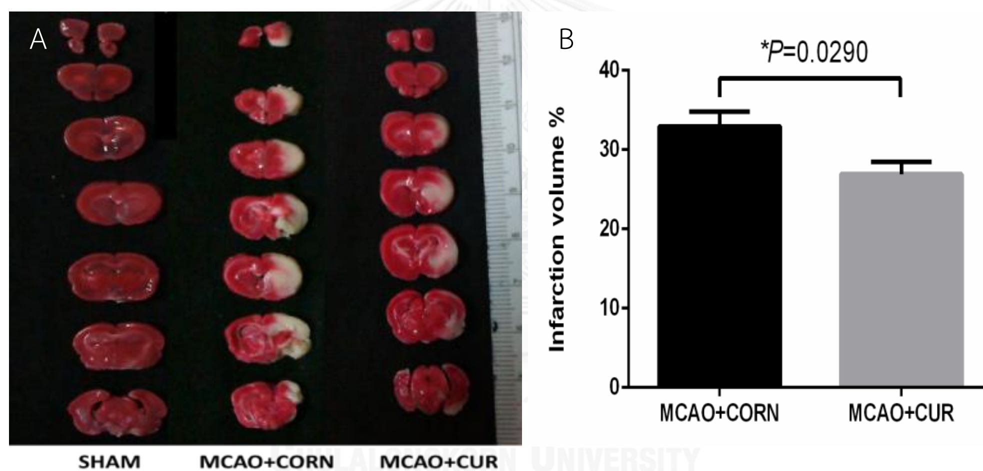



*Figure 4. 2 Infarction volume among different groups. Infarction volume was obvious larger in MCAO+CORN group than MCAO+CUR group; no infarction volume was detected in SHAM group (A) (n=1 rat/group); the statistical analysis results confirmed that there was significant difference between MCAO+CORN and MCAO+CUR groups (B) (n=7 rats/group).*

4.3 Physiological parameters

Owing to the slightly alteration of blood gas and blood pressure could directly influence the cerebral microcirculation, especially exposure of pial. To maintain arterial blood gas and mean artery blood pressure (MABP) within normal range (pH: 7.35-7.45, PaCO2: 35-45 mm Hg, PaO2: 80-120 mm Hg MABP: 80-120 mm Hg) among different groups after MCA occlusion. Left CCA blood was collected for detecting blood gases with blood-gas analyzer (i-STAT® analyzer, Abbott Company, USA). And artery blood pressure was measured with polygraph system (Nihon Kohden, Japan). (Table 4.1)

Table 4.1. The physiological parameters

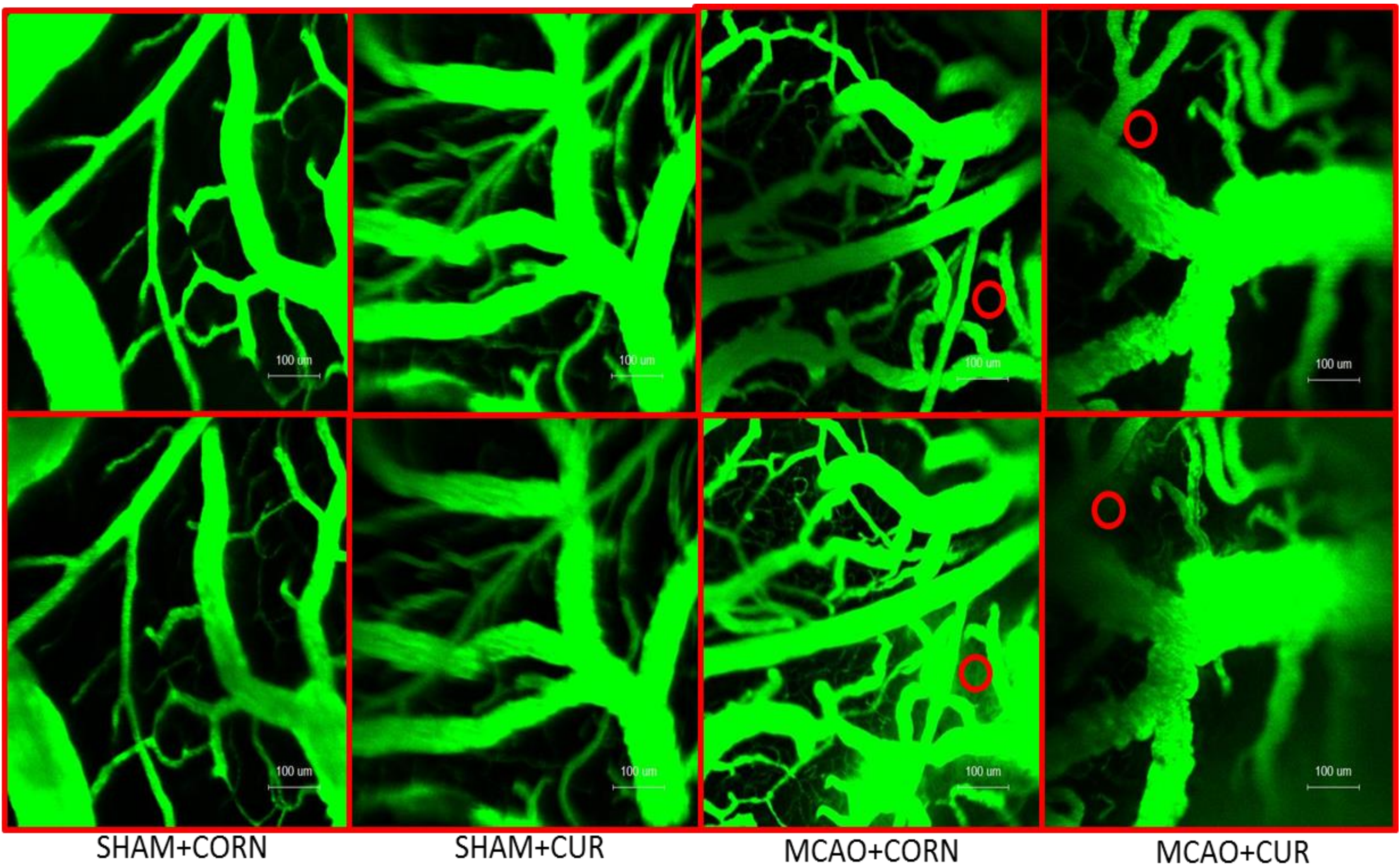
| | | SHAM+CORN | SHAM+CUR | MCAO+CORN | MCAO+CUR |
|---|---|---|---|---|---|
| MABP | Before | 98.1±6.3 | 89.1±11.2 | 87.5±11.8 | 92.7±14 |
| | After | 102.3±11.6 | 95.3±9.8 | 95.7±13.3 | 90.5±12.2 |
| PaO2 | Before | 136.5±19.3 | 114.3±10.2 | 109.6±16.7 | 97.4±19 |
| | After | 128.1±14.2 | 122±15.4 | 125.7±21.9 | 115.9±22.1 |
| PaCO2 | Before | 44.1±5.2 | 51.2±10.3 | 60.9±13.2 | 63±13.4 |
| | After | 47.3±8.1 | 46.7±5.6 | 54.7±5.06 | 53.4±6.5 |
| pH | Before | 7.37±0.11 | 7.41±0.91 | 7.30±0.06 | 7.32±0.94 |
| | After | 7.35±0.21 | 7.36±0.09 | 7.31±0.03 | 7.39±0.04 |

*Table 4. 1 The physiological parameters results. There was no significant differences among each group, no matter before or after BBB detection.*

4.4 BBB leakage detection

As shown in Figure 4.3.A, no obvious FITC leakage was observed outside the lumen of the capillary network, at either 0 or 10 min post FITC injection. For MCAO groups, the FITC leakage was stronger at 10 min than the results of 0 min after FITC injection in both curcumin (0.8494 ± 0.5312) and corn oil treated MCAO groups (0.7963 ± 0.3099). The FITC leakage was shown in white circle areas. However, the results shown in Figure 4.3.B, indicated that the MCAO+CUR group (7.185 ± 1.494) had significantly lower BBB disruption compared with the MCAO+CORN group (13.08 ± 2.394, $^*P<0.05$).

A



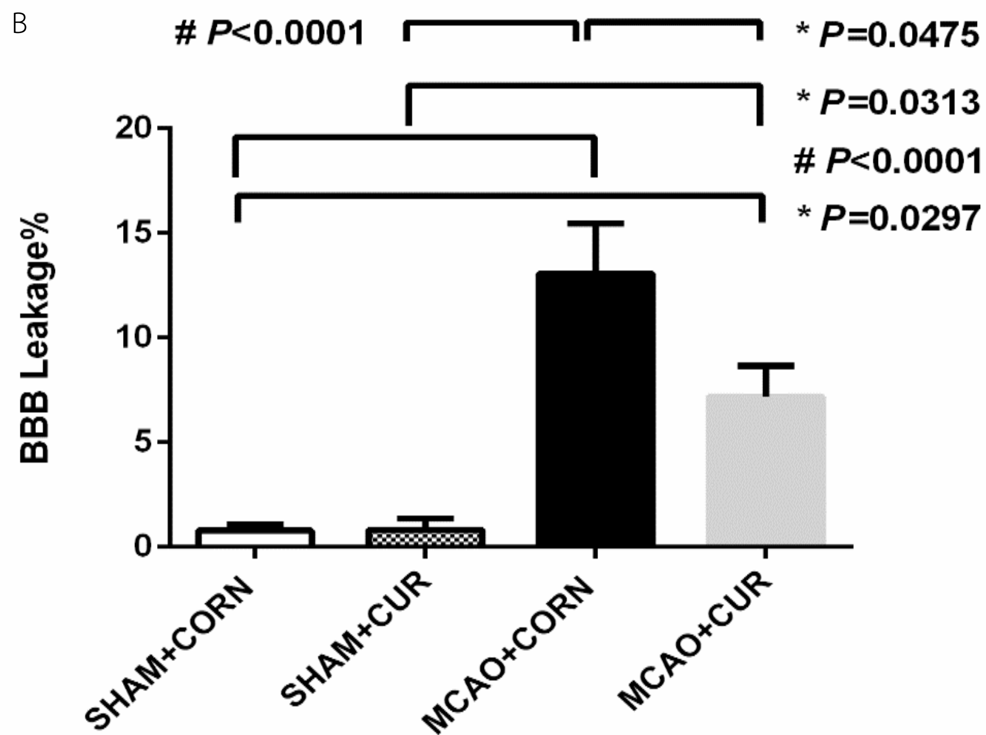



*Figure 4. 3 The BBB leakage (A and B). The FITC-Dx-250 flux across the capillary venule (20-40 μm) wall was observed through intravital confocal microscopy at 0 and 10 min after FITC i.v. injection. For the SHAM group, no obvious difference in the FITC flux was observed at 10 min compared with 0 min post FITC injection. However, the FITC leakage at 10 min was obviously stronger than that at 0 min post FITC injection, as shown in Figure 4. 3 A. The white circles represent the regions of interest; scale bar = 100 μm. The statistical results are shown in Figure 4.3 B. The BBB leakage (%) was significantly enhanced in the MCAO+CORN group compared with the SHAM group. In the MCAO+CUR group, the BBB leakage (%) was significantly lower than that in the MCAO+CORN group (n=5 rats/group).*

## 4.5 Water content

After sacrificing the rat, the brain was removed and divided into 2 parts, and the wet and dry weight were obtained before and after drying for 48 hours in an oven at 70 °C. The following formula was used to calculate the percentage of water content.

Water content (%) = (Wet weight – Dry weight)/Wet weight ×100%

The water content (%) was significantly higher in the MCAO+CORN group (68.2 ± 1.118) compared with either the SHAM+CORN (54.12 ± 2.699, **$P < 0.0001$) or the SHAM+CUR (51.91 ± 1.545, #$P < 0.0001$) group. In addition, curcumin treatment (58.83 ± 1.397, *$P < 0.05$) significantly prevented brain edema compared with the MCAO+CORN group (Figure 4.4).

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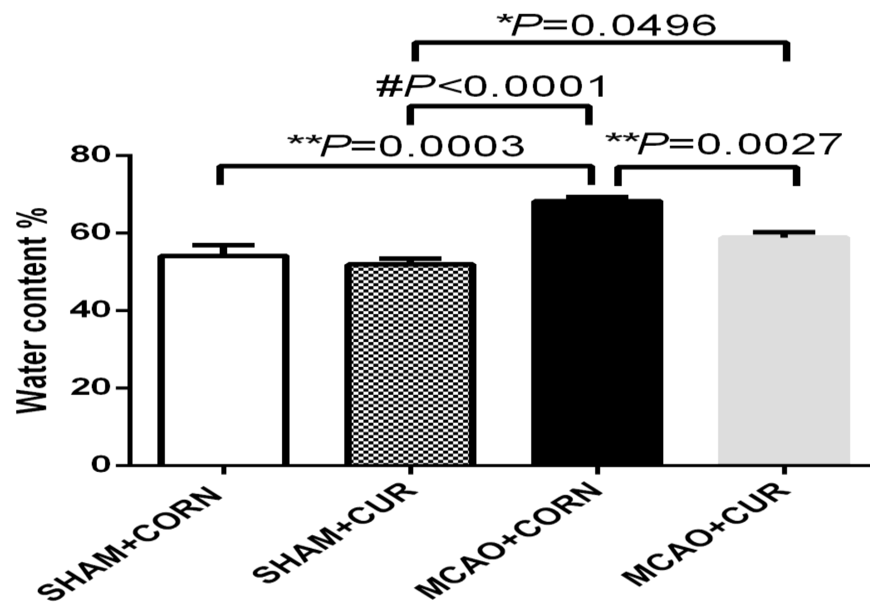


*Figure 4. 4 Effect of curcumin on the brain water content (%) at 24 hours post-reperfusion. Water content (%) was significantly higher in MCAO+CORN group vs. SHAM-operation group (n=3 rats/group). The results in the MCAO+CUR group were significantly lower compared with the MCAO+CORN group (n=5 rats/group for MCAO groups).*

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4.6 MDA level

To examine antioxidant effect of curcumin on stroke, the MDA level (Lipid peroxidation) of the affected brain tissue (3-6 mm posterior to bregma) was measured by TBARS assay (Dawn-Linsley et al., 2005). The MDA level was significantly elevated in MCAO+CORN group compared with SHAM groups (#$P$<0.0001). However, the MDA level was significantly lower in MCAO+CUR group (0.8933 ± 0.04890) than MCAO+CORN group (1.173 ± 0.09964, *$P$<0.01) (Figure 4.5).

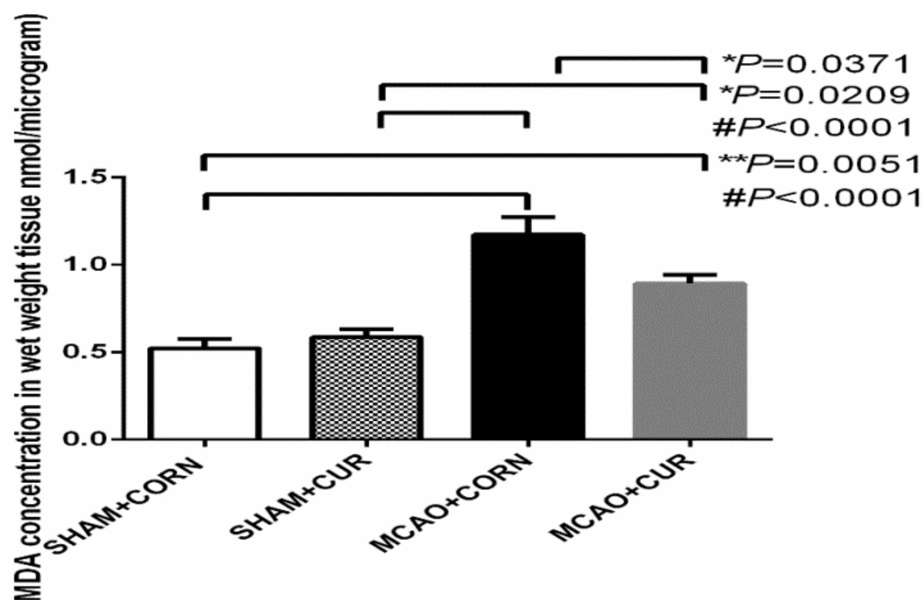


*Figure 4. 5 Effect of curcumin on MDA level in affected hemisphere 24 hours post-reperfusion. The MDA level was significantly higher in the MCAO+CORN group vs. the SHAM group. In addition, the MDA level was significantly lower in the MCAO+CUR group vs. the MCAO+CORN group (n=5 rats/group).*

4.7 The image of hematoxylin and eosin (H&E) staining

To detect the underlying mechanism from the level of proteins, the immunohistochemistry measurement was used. H&E staining was used to locate the injury and non-injury side, as shown for the non-injury side, the brain tissue was intact with uniform tissue background (A); the injury side as shown in B, there was abundant of coagulative necrosis and cavitation in the background (Figure 4.6).

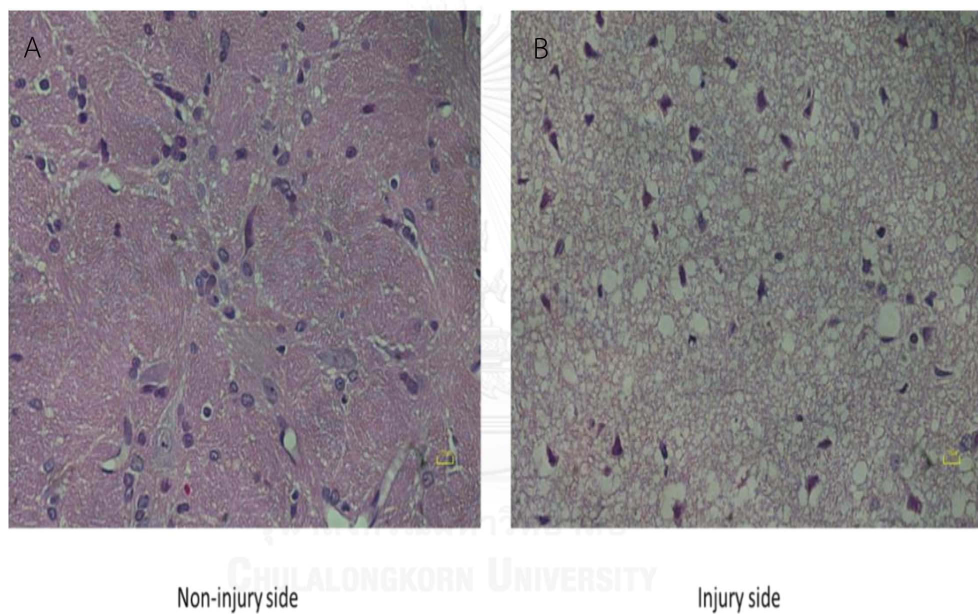


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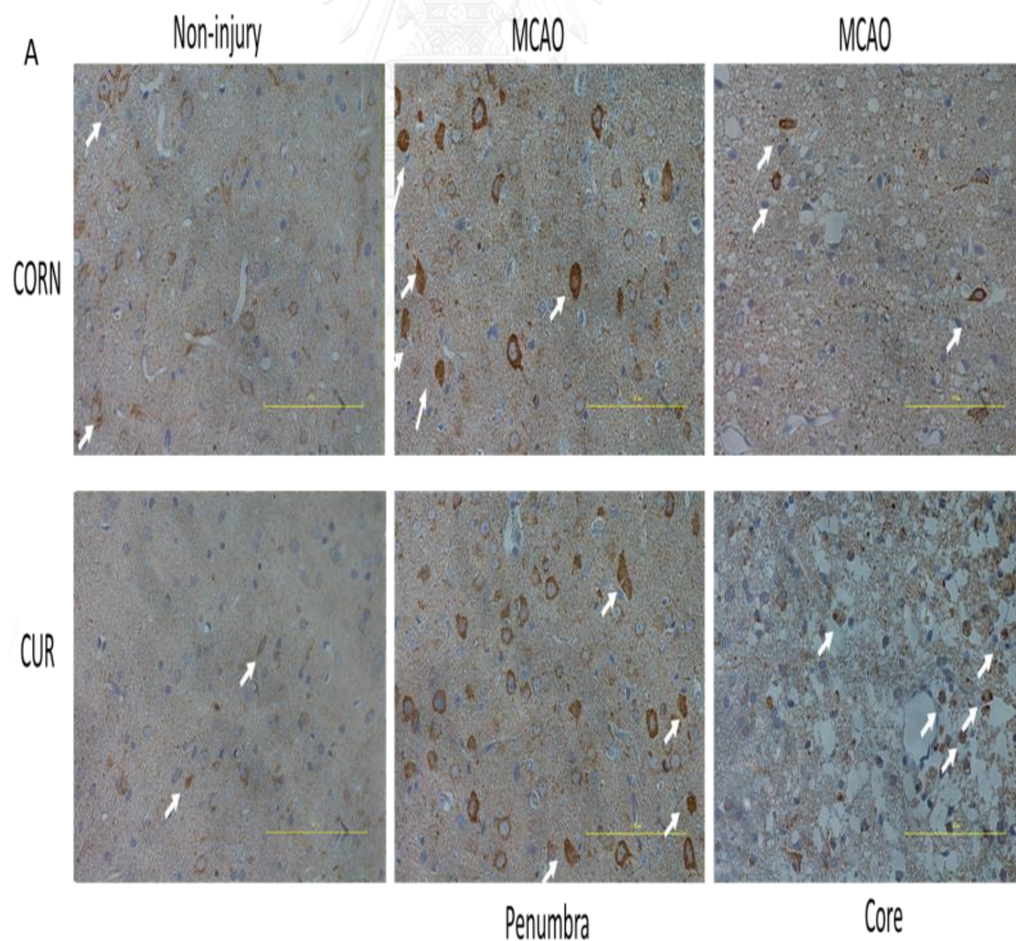
*Figure 4. 6 The H&E staining of brain tissue between non-injury and injury side (scale bar = 10 μm).*

4.8 Curcumin up-regulated the brain tissue Nrf2 expression in injury cortex area

From Figure 4.7, the positive immunoreactive cells were stained in brown color either in cytoplasm or nucleus pointed by white arrows (A). They were manually counted in penumbra, core and whole cortex areas (penumbra + core areas). There was no significant difference in nucleus positive Nrf2 cells number between injury and non-injury side of MCAO groups. However, for the total Nrf2 positive cells number (nucleus + cytoplasm), significantly higher results was observed in curcumin treated MCAO group than corn oil treated group in the whole cortex area (*$P$<0.05).



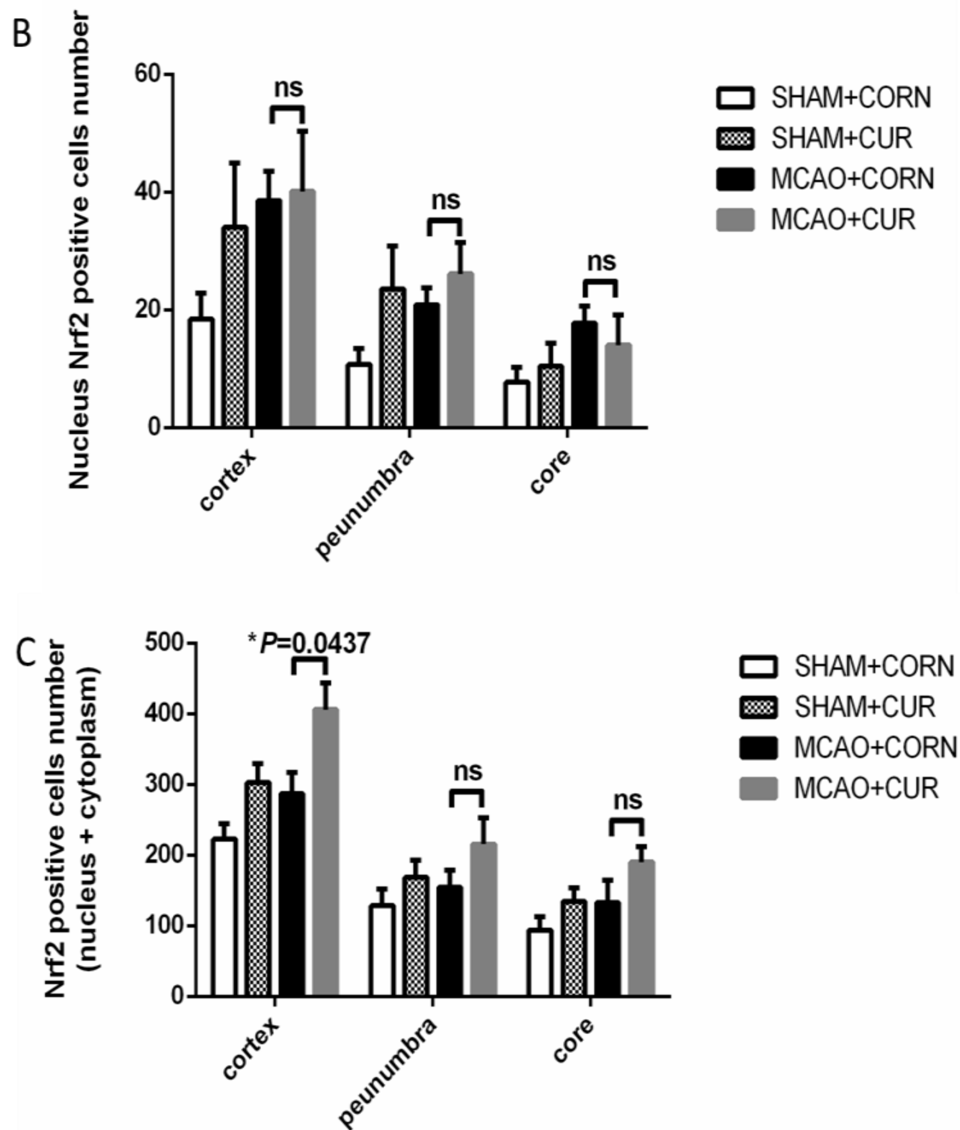



*Figure 4. 7 The effect of curcumin on the Nrf2 expression measured by immunohistochemistry. There was no obvious difference in positive Nrf2 staining among groups (A). The statistical analysis found that for whole cortex area, the total number of Nrf2 positive cells was significantly higher in curcumin treated MCAO group than corn oil treated group on injury side (B, C).*

4.9 CUR retarded the brain tissue ICAM-1 expression

The expression of ICAM-1 was detected by immunohistochemistry. The result was represented by integrated optical density (IOD)/total area in Figure 4.8. A. The result indicated that no matter in penumbra or core area, the positive stained areas were obviously stronger in injury side of MCAO groups than non-injury side, and curcumin treated MCAO group had lower expression of ICAM-1 than corn oil treated MCAO group. The positive staining was limited on the inner layer of capillary vessels (shown by white arrows). In non-injury side, no apparent positive staining area was visualized. Figure 4.8. B showed that no matter for the whole cortex, penumbra or core areas that the expression of ICAM-1 in injury hemisphere of MCAO+CORN group was significantly higher than non-injury side (** $P \leq 0.01$). However, curcumin could significantly prevented the over-expression of ICAM-1 compared with corn oil treated stroke group (** $P \leq 0.01$). The result of ICAM-1 expression was confirmed by histogram analysis with Image-Pro plus 6.0 software (Appendix C).

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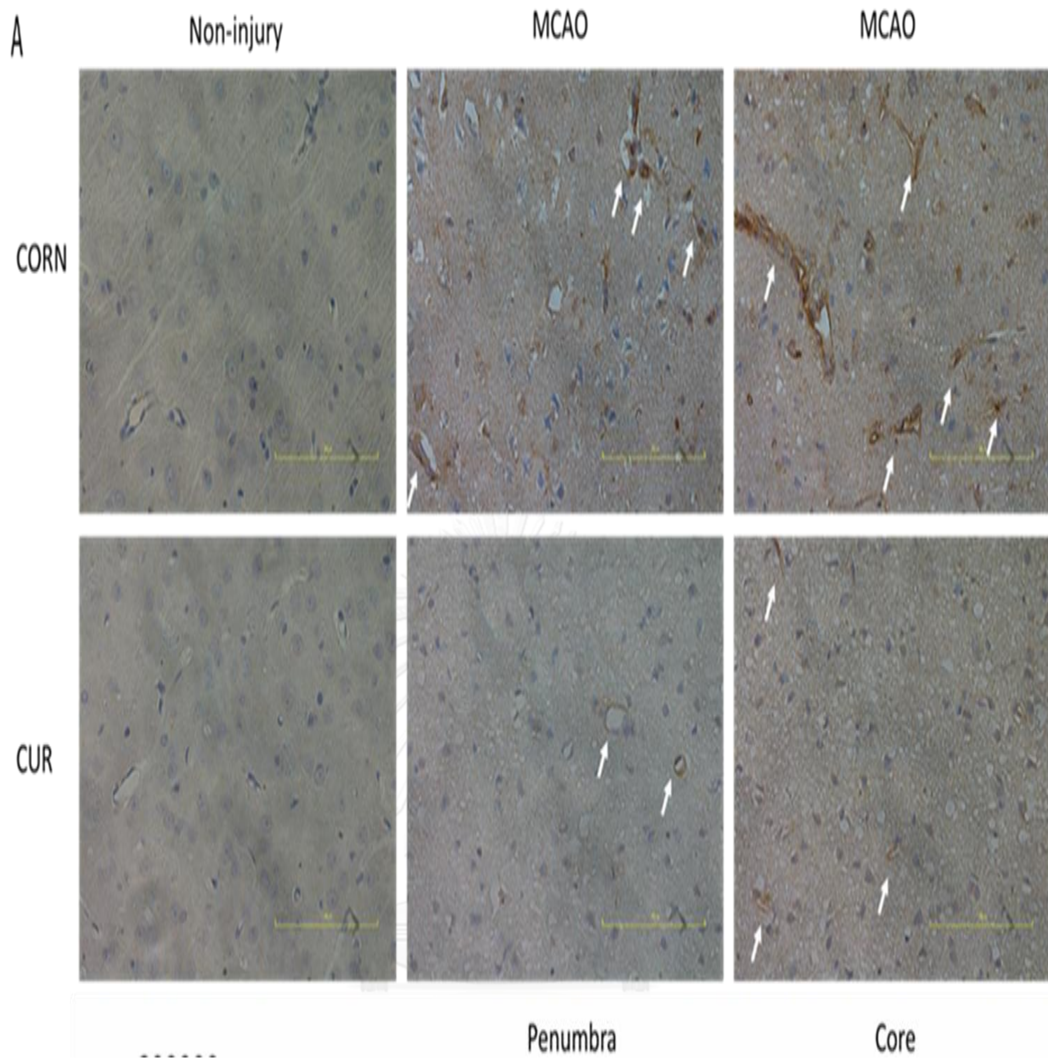


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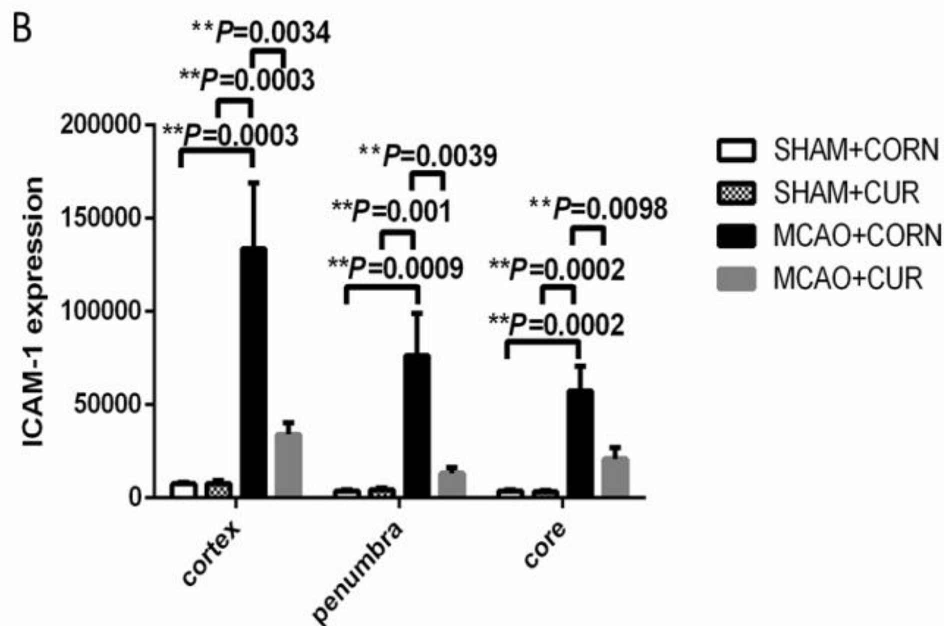


*Figure 4. 8 The effect of curcumin on the ICAM-1 expression measured by immunohistochemistry. The expression of ICAM-1 was higher in injury side of MCAO groups, the positive staining was only visualized on blood vessel area in Figure 4.8.A. The expression of ICAM-1 was significantly higher in corn oil treated MCAO groups vs. non-injury side (shown by white arrows). However, ICAM-1 expression was significantly lower in curcumin treated MCAO group vs. vehicles treated stroke group in no matter cortex, penumbra or core areas. (Figure 4.8.B)*

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4.10 CUR inhibited the brain tissue MMP-9 expression

Figure 4.9 A showed that there was no obvious MMP-9 positive staining (shown by white arrows) in non-injury side, but in both corn oil and curcumin treated MCAO groups the MMP-9 positive brown color staining were visualized in vicinity of capillary area especially in penumbra area. In Figure 4.9.B showed semi-quantification results of MMP-9 expression. In affected hemisphere of MCAO+CORN group, the IOD of MMP-9 was significantly higher than non-injury side (*$P \le 0.05$, **$P \le 0.01$, #$P \le 0.0001$). However curcumin treatment could significantly inhibited the up-regulation of MMP-9 *vs*. vehicles treated stroke group in whole cortex area (**$P \le 0.01$). The result of MMP-9 expression was confirmed by histogram analysis with Image-Pro plus 6.0 software (Appendix C).

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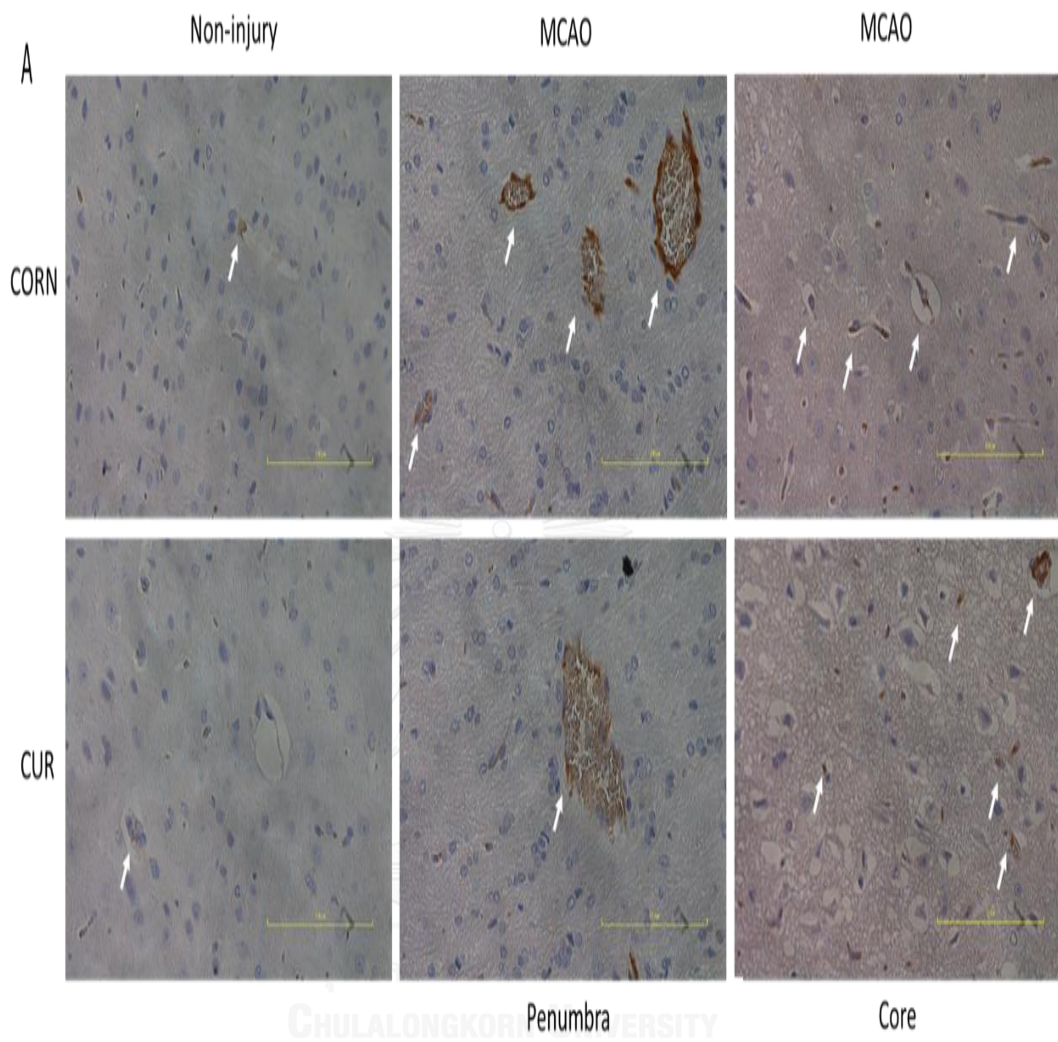


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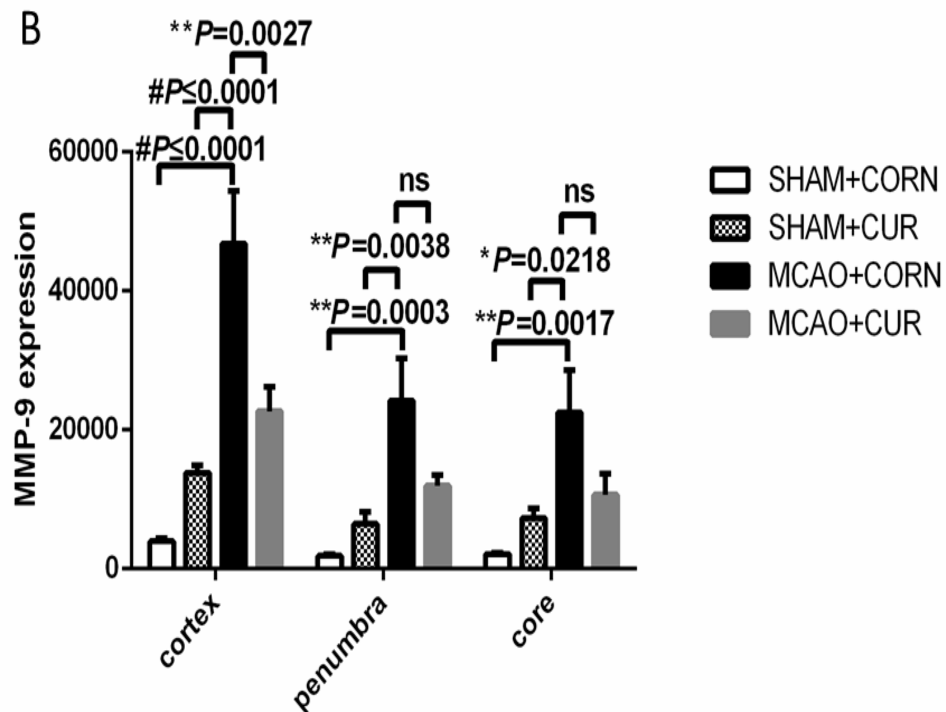
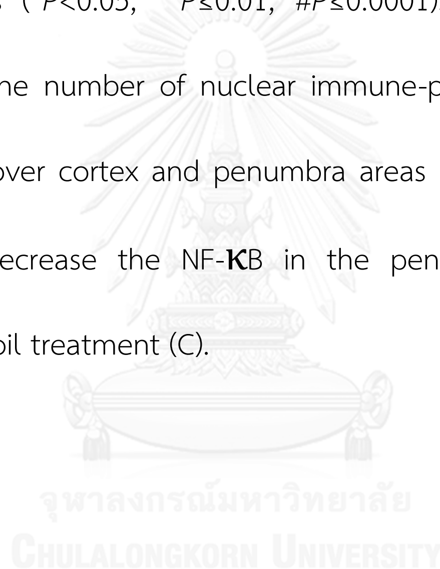
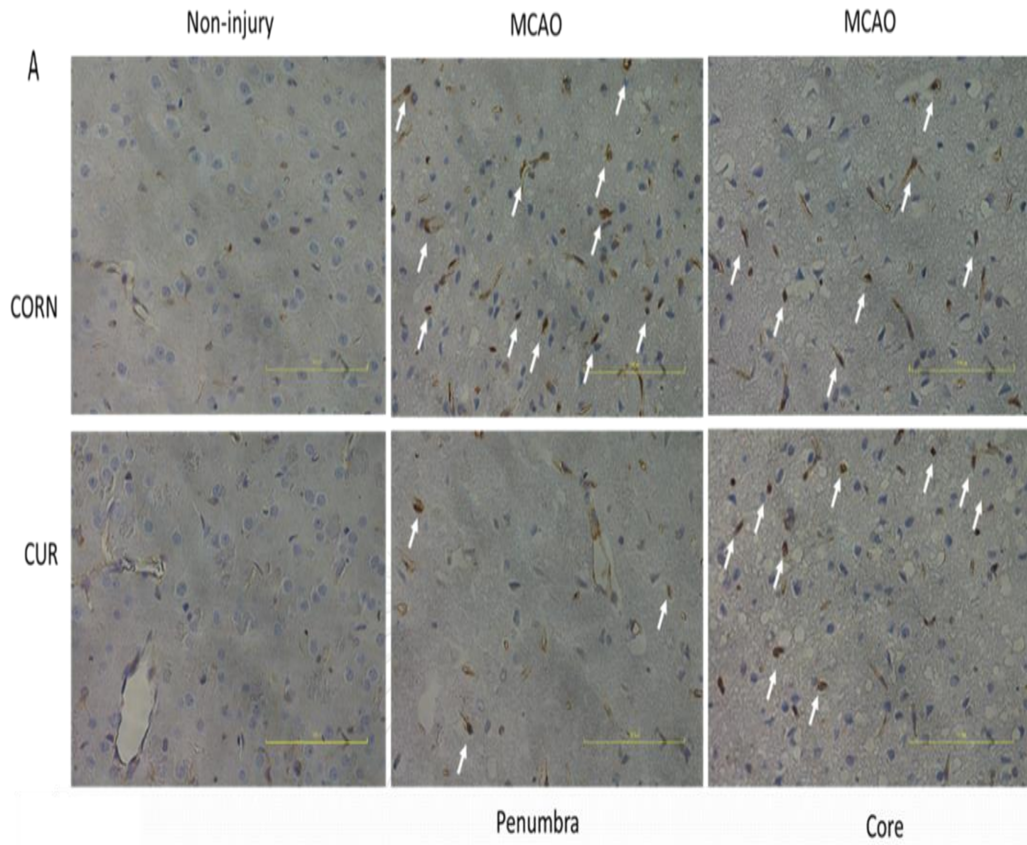


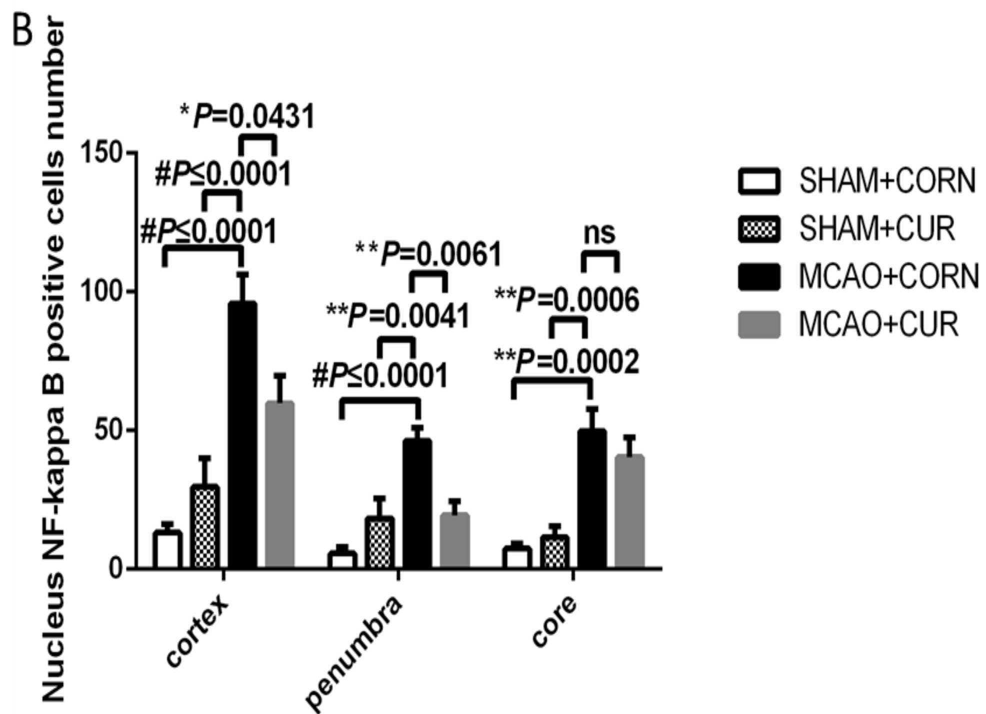

*Figure 4. 9 The effect of curcumin on the MMP-9 expression detected by immunohistochemistry. The positive staining of MMP-9 was visualized in MCAO affected sides, indicated by white arrows, and located in capillary area (A). The expression of MMP-9 was significantly higher in injury side of MCAO+CORN group vs. non-injury side* (*$P \le 0.05$, **$P \le 0.01$, #$P \le 0.0001$). *For injury side, with curcumin administration, the level of MMP-9 was significantly lower vs. corn oil treated group (B)* (**$P \le 0.01$).

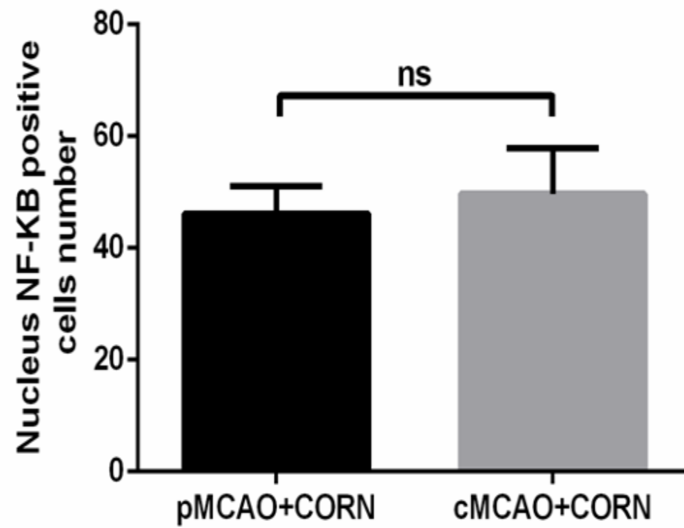
4.11 CUR reduced the brain tissue NF-kappa-B expression

Figure 4.10.A showed the pictures of NF-kappa-B (p65) unclear positive staining by immunohistochemistry. For non-injury side, no obvious immune-positive cells were observed, whereas, increased nuclear positive staining cells presented in MCAO group, as indicated by white arrows. Figure 4.10.B showed that nucleus positive cells in injury side of MCAO group was significantly more than non-injury side over all three areas (*$P<0.05$, **$P\leq0.01$, #$P\leq0.0001$). Curcumin treatment could significantly reduce the number of nuclear immune-positive cells as compared to MCAO+CORN group over cortex and penumbra areas (*$P<0.05$, **$P\leq0.01$). Curcumin could significantly decrease the NF-$\kappa$B in the penumbra area than core area compared with corn oil treatment (C).




Non-injury
MCAO
MCAO
A
CORN
CUR
Penumbra
Core


B
Nucleus NF-kappa B positive cells number
150
100
50
0
*P=0.0431
#P≤0.0001
#P≤0.0001
**P=0.0061
**P=0.0041
#P≤0.0001
ns
**P=0.0006
**P=0.0002
cortex
penumbra
core
SHAM+CORN
SHAM+CUR
MCAO+CORN
MCAO+CUR



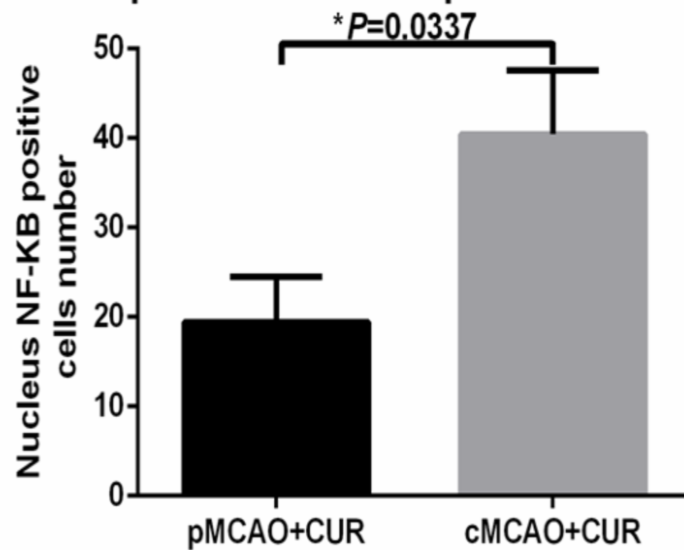




*Figure 4. 10 Effect of curcumin on the nucleus NF-kappa-B (p65) expression checked by immunohistochemistry. The nuclear positive staining cells of NF-kappa-B in different groups as indicated by white arrows; no obvious positive staining in non-*

_injury side was visualized; the positive staining cells number in curcumin treated MCAO group was less than corn oil treated MCAO group in injury side over penumbra area (A). The nuclear positive staining cells of NF-kappa-B in jury side of MCAO was significantly higher vs. non-injury side over whole cortex, penumbra and core areas (*$P<0.05$, **$P\leq0.01$, #$P\leq0.0001$). Curcumin could significantly reduce the NF-kappa-B (p65) nuclear expression vs. corn oil treated MCAO group over cortex and penumbra areas (*$P<0.05$, **$P\leq0.01$) (B)._ Curcumin significantly decreased the NF-κB in the penumbra area than core area compared with corn oil treatment (C).

4.12 CUR prevented the brain tissue caspase-3 expression

Figure 4.11.A showed that increased nucleus caspase-3 positive staining cells in MCAO groups indicated by brown color staining cells (pointed by white arrows). Statistical analysis in Figure 4.11.B found that for the whole cortex and penumbra areas, the cells number of nucleus positive caspase-3 was significantly enhanced in injury side compared with non-injury side (**$P \leq 0.01$, #$P \leq 0.0001$). Whereas curcumin could significantly block activation of nuclear caspase-3 in penumbra area compared with vehicle treated MCAO group (*$P<0.05$). The distribution of caspase-3 was significantly expressed higher in penumbra area than core area for MCAO+CORN group. Curcumin could significantly prevent caspase-3 in penumbra area than corn oil in figure 4.11.C.

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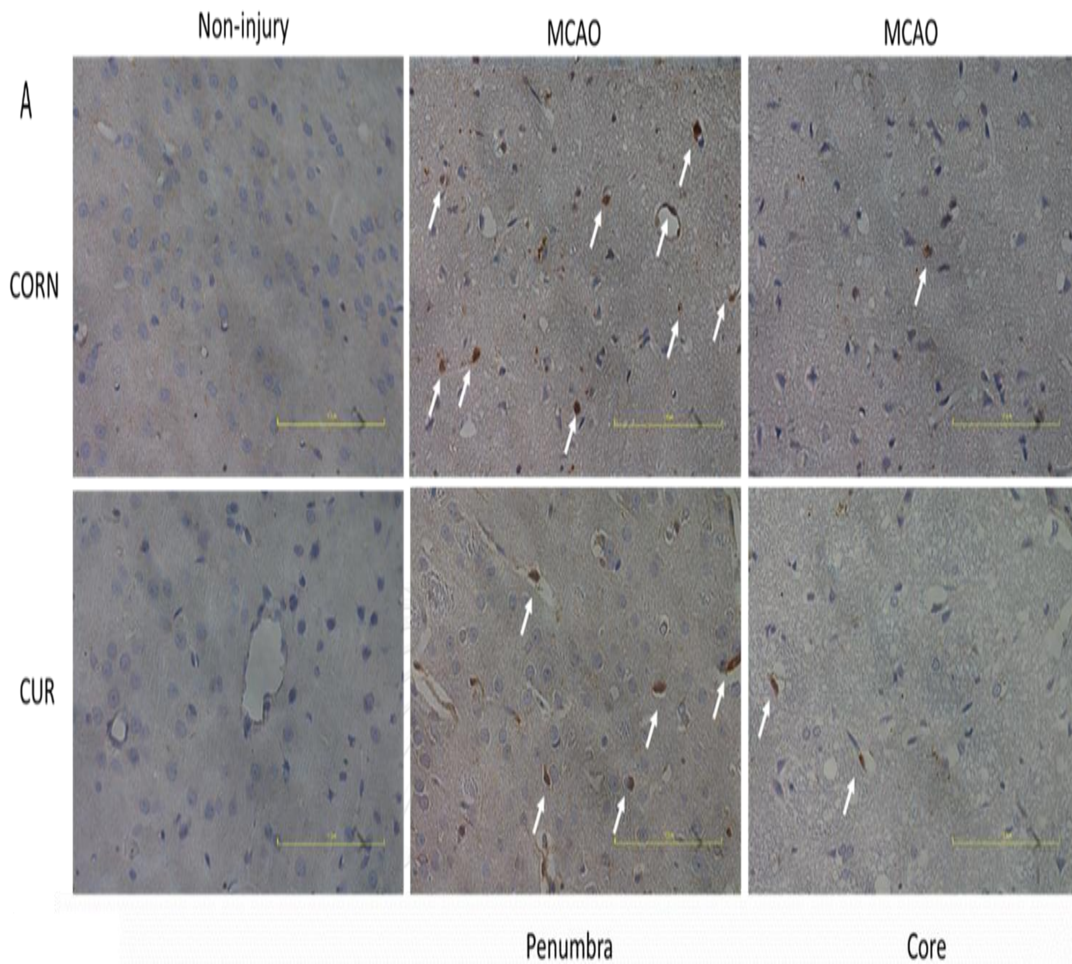
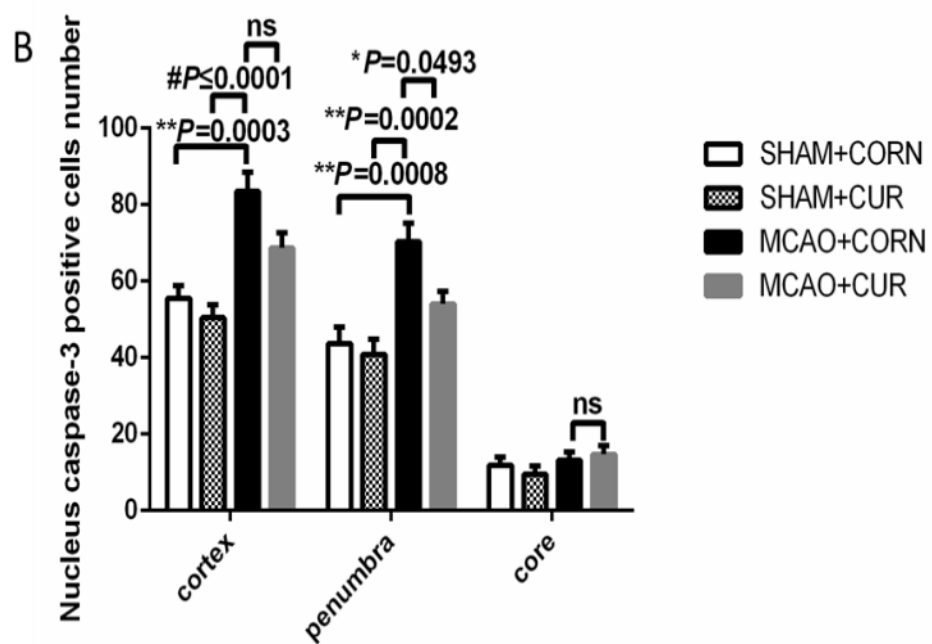
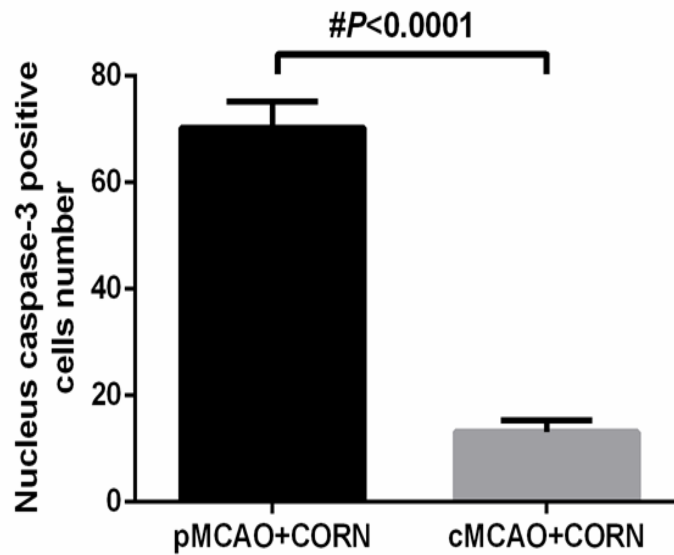

Non-injury
MCAO
MCAO
A
CORN
CUR
Penumbra
Core


B
ns
#P≤0.0001
**P=0.0003
*P=0.0493
**P=0.0002
**P=0.0008
ns
Nucleus caspase-3 positive cells number
100
80
60
40
20
0
cortex
penumbra
core
SHAM+CORN
SHAM+CUR
MCAO+CORN
MCAO+CUR



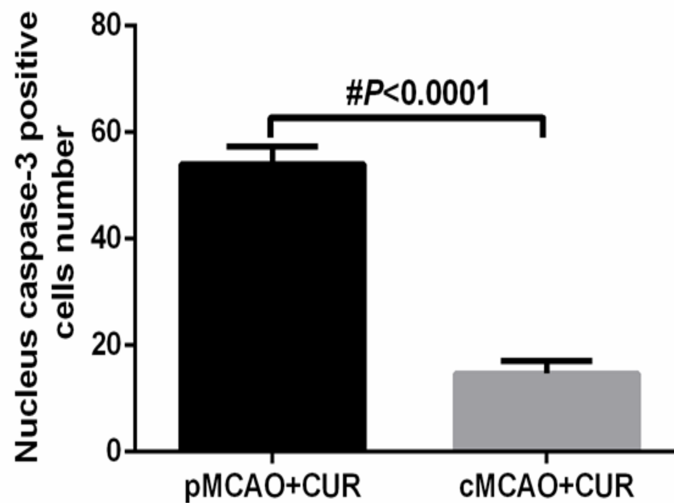




*Figure 4. 11 The effect of curcumin on the caspase-3 expression detected by immunohistochemistry assay. The positive nucleus staining cells number of caspase-3 in different groups as labeled by white arrows (A). The nucleus positive staining cells number of caspase-3 was significantly higher in injury side of MCAO group for*

*whole cortex and penumbra areas vs. non-injury side (**$P \le 0.01$, #$P \le 0.0001$). Whereas curcumin could significantly reduce the caspase-3 expression vs. corn oil treated MCAO group only in penumbra area (B) (*$P < 0.05$). The distribution of caspase-3 was significantly expressed higher in penumbra area than core area for MCAO+CORN group. Curcumin could significantly prevent caspase-3 in penumbra area than corn oil (C).*

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4.13 The correlation between parameters

The Pearson's correlation analysis was conducted between the mean values of each parameter from different groups.

4.13.1 The BBB leakage & edema

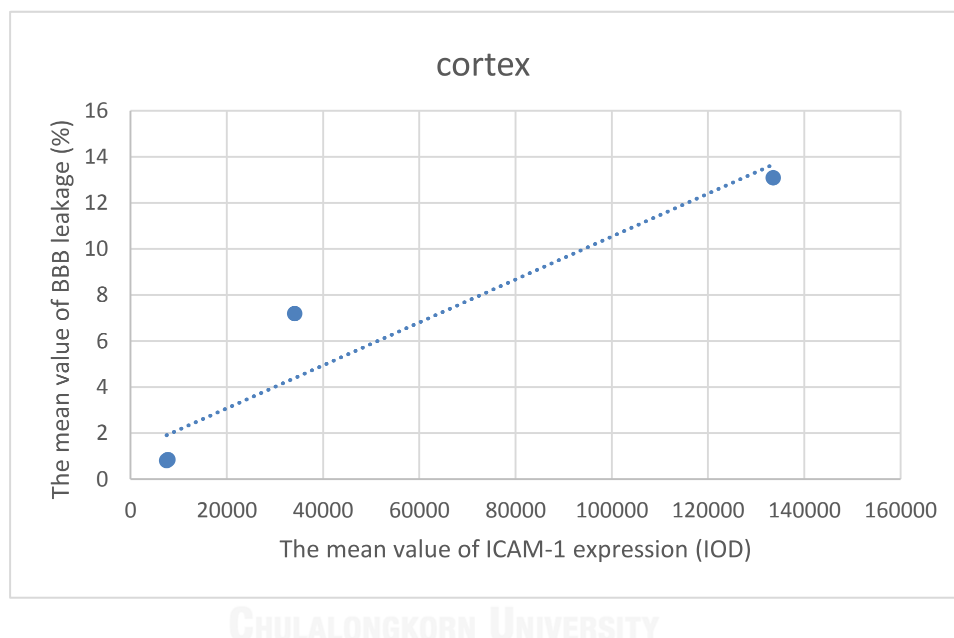


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*Figure 4. 12 The correlation between mean values of BBB leakage and edema in the cortex area. Pearson's correlation coefficient is r=0.982 (P=0.018).*

4.13.2 The infarction volume & neurological dysfunction

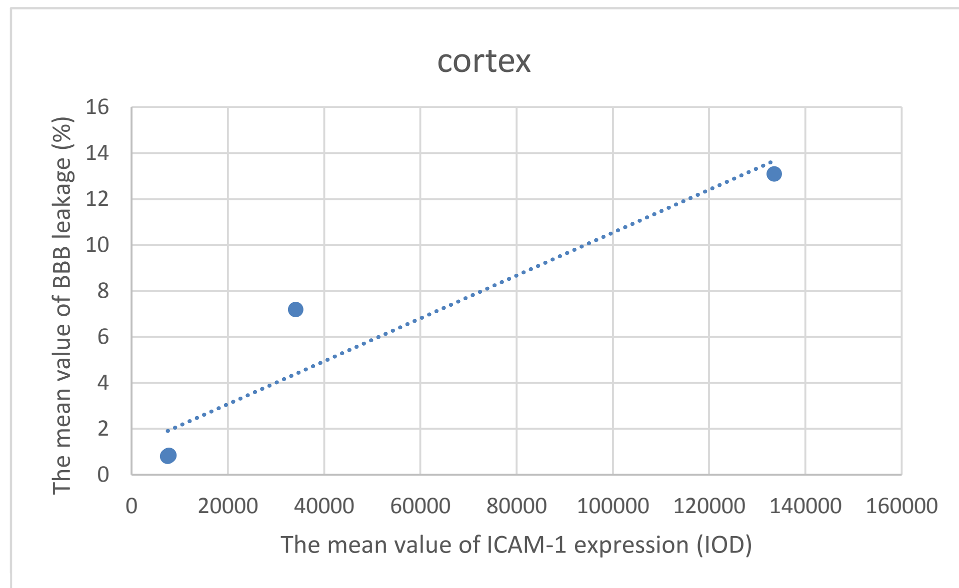


*Figure 4. 13 The correlation between mean values of infarction volume and neurological deficit scores in the cortex area. Pearson's correlation coefficient is r=0.995 (P=0.005).*

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4.13.3 The level of ICAM-1 (IOD) & BBB leakage (%)

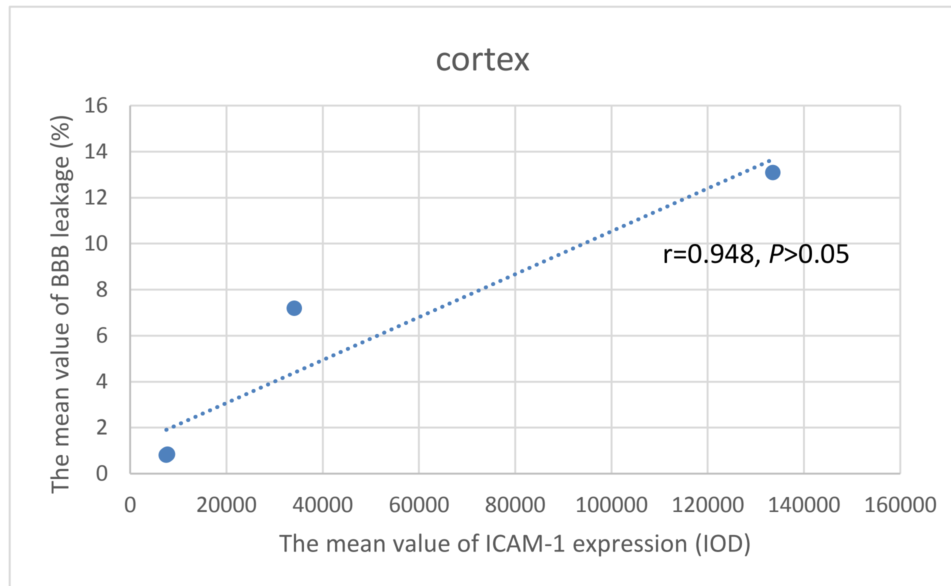


*Figure 4. 14 The correlation between mean values of ICAM-1 and BBB leakage (%) in the cortex area. Pearson's correlation coefficient is r=0.948 (P=0.052).*

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4.13.4 The level of ICAM-1 (IOD) & edema (%)

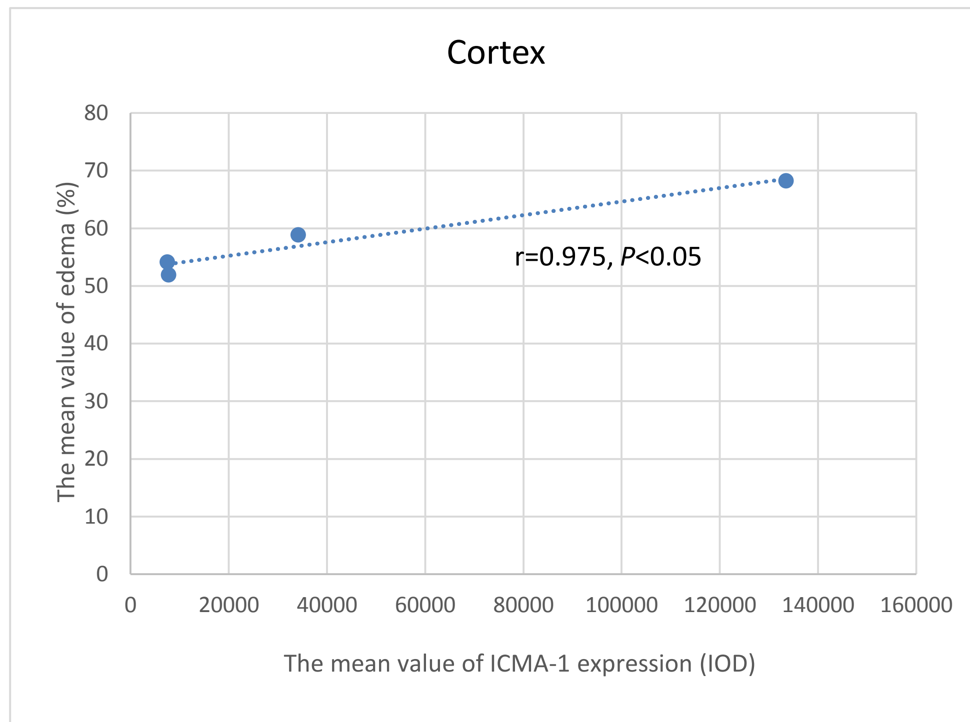


*Figure 4. 15 The correlation between mean values of ICAM-1 and edema in the cortex area. Pearson's correlation coefficient is r=0.975 (P=0.025).*

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4.13.5 The level of MMP-9 (IOD) & BBB leakage (%)

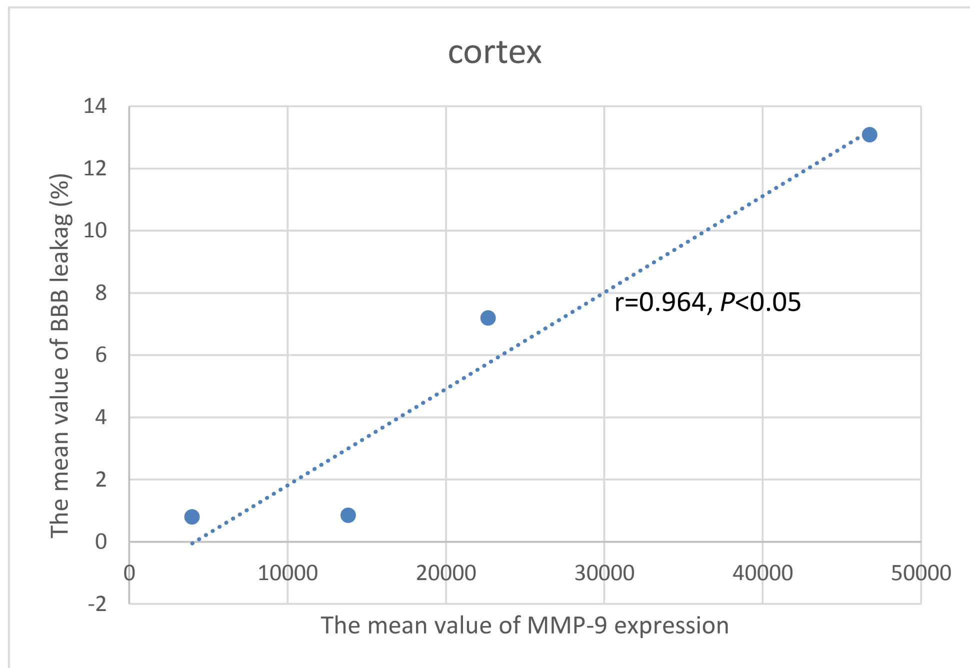


*Figure 4. 16 The correlation between mean values of MMP-9 and edema in the cortex area. Pearson's correlation coefficient is r=0.964 (P=0.036).*

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4.13.6 The level of ICAM-1 (IOD) & MMP-9 (IOD)

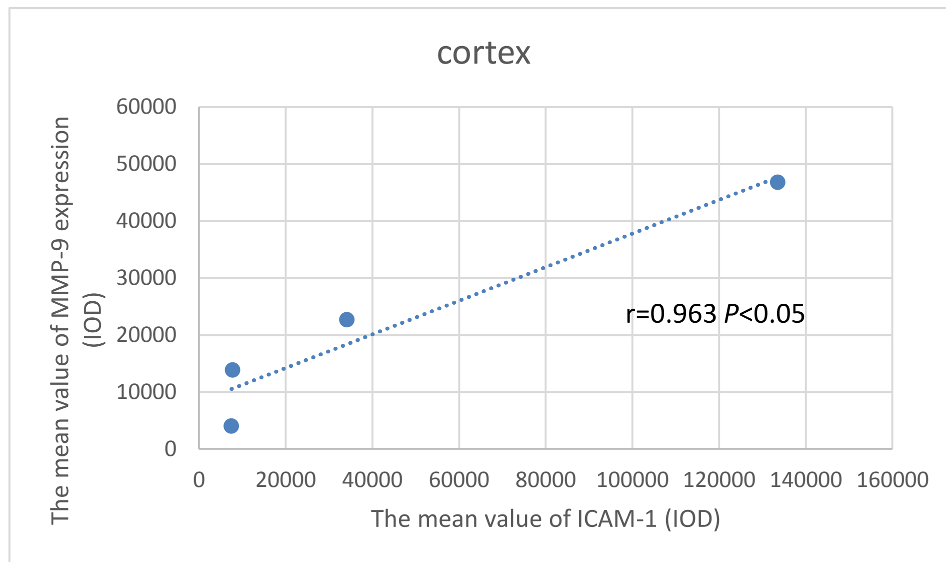


*Figure 4. 17 The correlation between mean values of MMP-9 and ICAM-1 in the cortex area. Pearson's correlation coefficient is r=0.963 (P=0.037).*

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4.13.7 The level of nucleus NF-**K**B (cells number) & ICAM-1 (IOD)

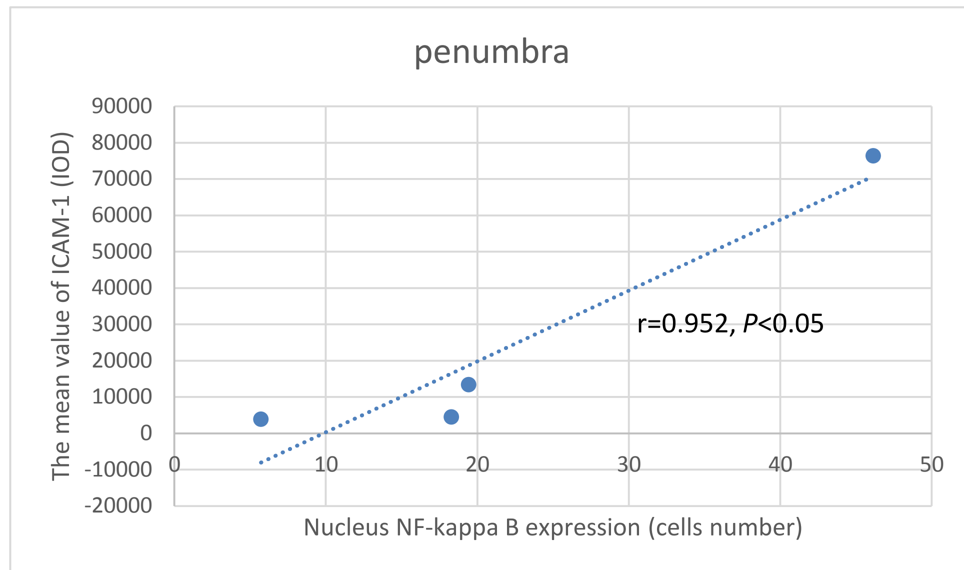


*Figure 4. 18 The correlation between mean values of NF-**K**B and ICAM-1 in the penumbra area. Pearson's correlation coefficient is r=0.952 (P=0.048).*

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4.13.8 The level of nucleus NF-ΚB (cells number) & MMP-9 (IOD)

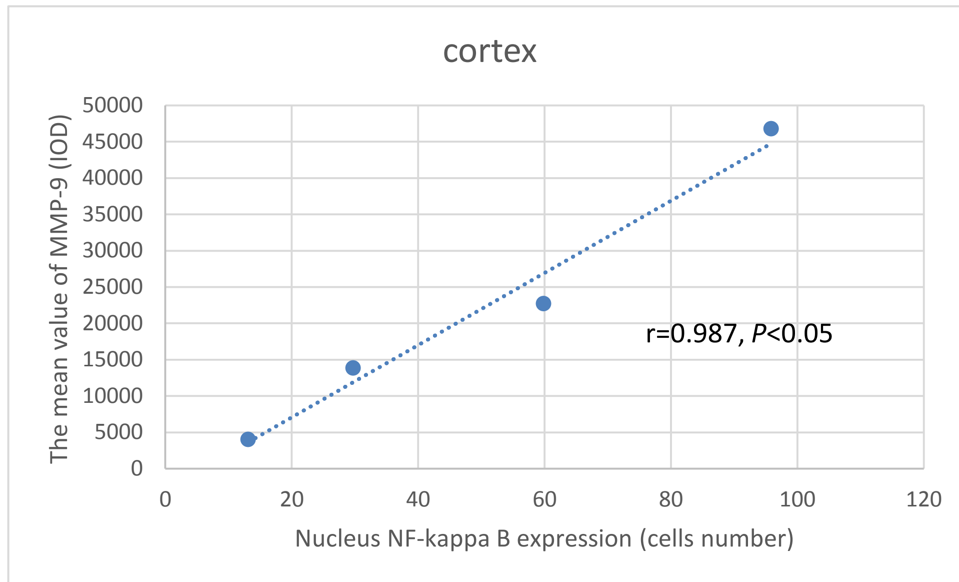


*Figure 4. 19 The correlation between mean values of NF-ΚB and MMP-9 in the cortex area. Pearson's correlation coefficient is r=0.987 (P=0.013).*

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4.13.9 The cells number of nucleus caspase-3 & infarction volume

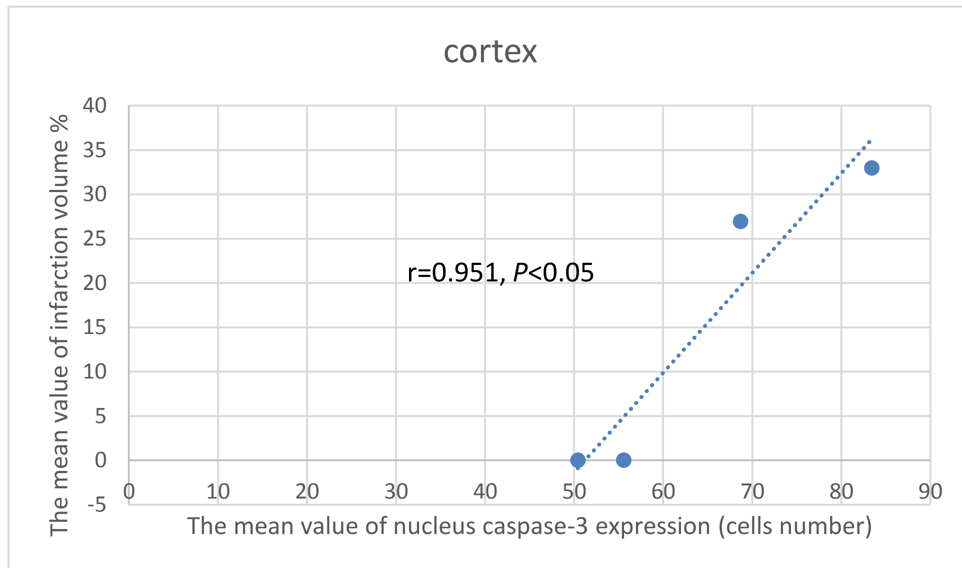


*Figure 4. 20 The correlation between mean values of caspase-3 and infarction volume in the cortex area. Pearson's correlation coefficient is r=0.951 (P=0.049).*

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4.13.10 The cells number of nucleus caspase-3 & edema

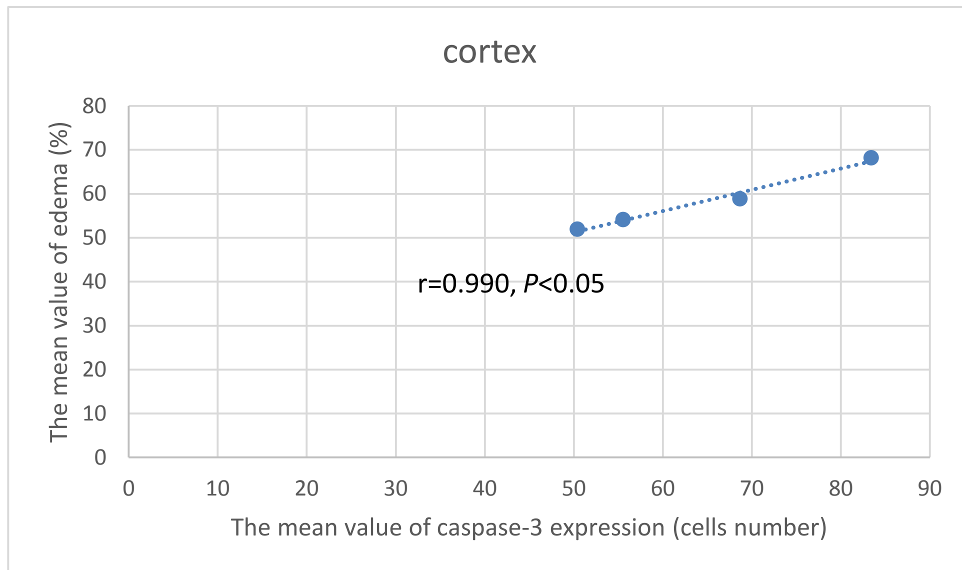


*Figure 4. 21 The correlation between mean values of caspase-3 and edema in the cortex area. Pearson's correlation coefficient is r=0.990 (P=0.010).*

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4.13.11 The cells number of nucleus caspase-3 & neurological deficit scores

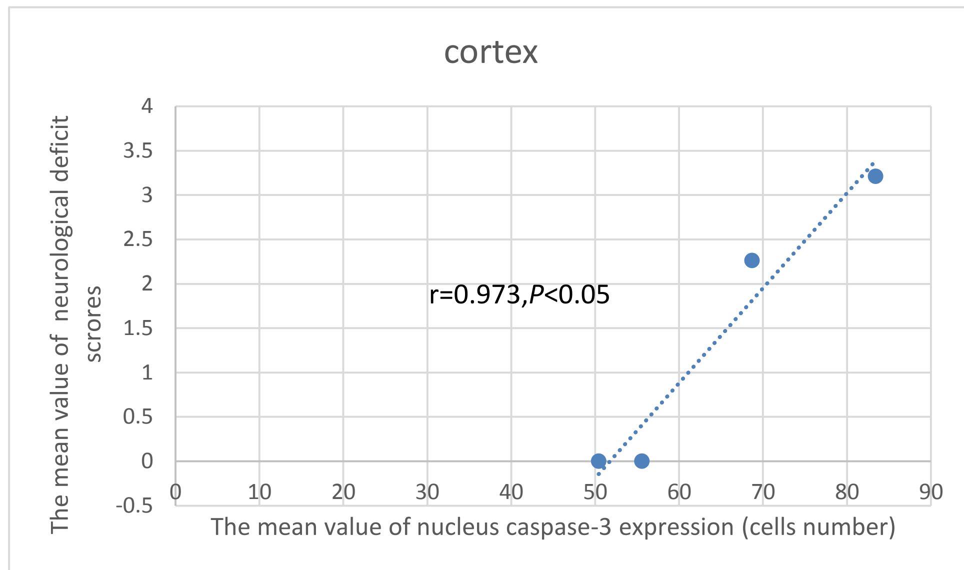


*Figure 4. 22 The correlation between mean values of caspase-3 and neurological deficit scores in the cortex area. Pearson's correlation coefficient is r=0.973 (P=0.027).*

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4.13.12 The cells number of nucleus caspase-3 & BBB leakage

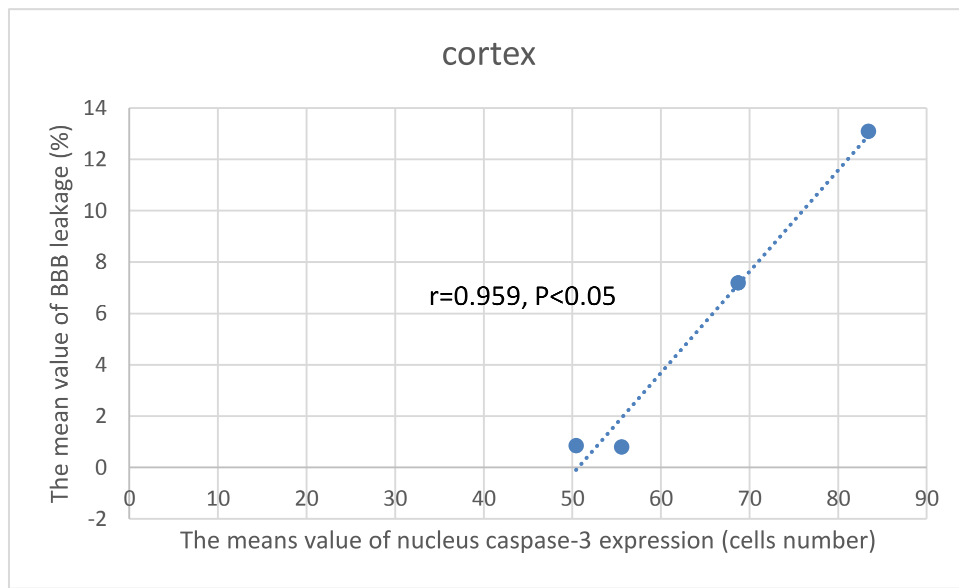


*Figure 4. 23 The correlation between mean values of caspase-3 and BBB leakage in the cortex area. Pearson's correlation coefficient is r=0.989 (P=0.011).*

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# CHAPTER 5

## DISSCUSSION

The results of the present study showed that curcumin exerted neuro-protective effects upon cerebral ischemia reperfusion damage, by preventing the deterioration of neurological dysfunction, infarction volume, brain edema and BBB disruption. The preventing mechanism might be associated with the down-regulation of NF-κB, caspase-3, ICAM-1 and MMP9 and the up-regulation of Nrf2 expression. To illustrate the content clearly, the discussion part was divided into two parts, the physiological characteristics of MCAO model and the protective effect of curcumin against cerebral I/R injury.

### 5.1 Biochemical and physiological characteristics of the MCAO rat model for I/R injury used in this study

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Cerebral ischemia stroke includes the global and focal ischemia [129]. For focal ischemia, the middle cerebral artery occlusion (MCAO) is the most common animal model used to mimic human ischemic stroke [129]. Several techniques have been established to induce MCAO model by ligation [143], electric cauterization [129], intraluminal filament occlusion [144], blood clot injection [145], or photochemical thrombosis [146]. However, among them, the intraluminal suture MCAO model induction is able to create the possibility of reperfusion, and reduce

the severity of trauma, complexity of operation and variations of model induction. According to our previous pilot study, the 1-hour MCAO with intraluminal suture was used for proving our research hypothesis. The MCAO model was confirmed by 2 criteria as illustrated in the method.

5.1.1 CBF examination

This could monitor the relative cerebral blood flow change on the surface of cortex. It is a well-accepted method to check the stroke model induction [137, 147, 148]. For adult rat, the craniotomy has necessary to be prepared over the MCA territory before continue observing of the CBF during whole operation. Even though our criteria for model induction was not that strict compare with Wang's criteria (lower than 30% for occlusion level). However, different lab could adjust their own criteria according to their lab condition, such as, Dang's report, the stroke inclusion criteria was set at lower than 50% for occlusion level [149]. In our study, 3 rats were subjected to CBF examination (Figure 3.6). Then 19%, 25% and 33% of CBF were obtained for 0 min post occlusion. Moreover, 75%, 68% and 80% were obtained for 20min post reperfusion.

5.1.2 Neurological deficit scores

MCA normally supplies blood flow to the majority of lateral surface of the cortex, except the superior potion of parietal lobe (ACA) and the inferior portion of the temporal lobe and occipital lobe (PCA). In addition, MCA also supplies part of the

internal capsule and basal ganglia, which are belong to striatum [150-153]. The MCA territory controls the motor function of the body [131]. Once the MCA is blocked, the related motor function will be impaired. Many researchers have reported that, this method can be used as the inclusion criteria for stroke group [17, 154-156]. The neurological dysfunction was scaled by 5 points scores as mentioned in the methodology. The score of stroke group should be lower than 5 and higher than 0. In our results before reperfusion, the neurological deficit scores of MCAO group (3.00 ± 0.1646 and 3.00 ± 0.1510) was significantly higher than the scores of SHAM group (0), 1 h after MCAO model induction (Figure 3.7).

By using above checking methods, which effectively guaranty our MCAO model induction. Then the biochemical and physiological characteristics of the MCAO rat model were detected in the following experiments.

The neurological dysfunction is one of the critical symptoms of stroke. It was detected again after 24 hours reperfusion (before sacrifice rats). Our results illustrated that the neurological deficit scores of MCAO group (3.207 ± 0.2450) was significantly higher than SHAM group (0) (Figure 4.1). This result was in line with the research of Panahpour who also used same model for study (1 hour MCAO plus 24 hours reperfusion) [134]. In our study, the rat normally demonstrated decreased resistance to lateral push and making circle to the non-injury side, which is quite similar as described in Engel's study, upon 60 min MCAO, the rat normally had mild

behavioral deficit results and the scores were lower than 4 [128]. Therefore, the symptoms supported that brain damage was induced by MCAO.

As shown in Table 5.1, the biochemistry and physiological characteristics of SHAM and MCAO rats were summarized.

| Rat group | Body weight | | Neurological deficit scores | %infarction volume | %BBB leakage, n=5 | %water content | MDA (nmol/μg), n=5 |
|---|---|---|---|---|---|---|---|
| | before lab | after lab | | | | | |
| SHAM+CORN | 267.8±3.854, n=9 | 253.2±4.245, n=9 | 0±0#, n=9 | 0±0#, n=1 | 0.7963±0.3099# | 54.12±2.699**, n=3 | 0.5226±0.05231# |
| SHAM+CUR | 274.7±4.64, n=9 | 261.3±4.699, n=9 | 0±0#, n=9 | 0±0#, n=1 | 0.8494±0.531# | 51.91±1.545#, n=5 | 0.5872±0.04584# |
| MCAO+CORN | 274.2±2.132, n=24 | 248.1±3.416, n=24 | 3.207±0.2450, n=29 | 32.96±1.871, n=7 | 13.08±2.394 | 68.2±1.118, n=5 | 1.173±0.09964 |
| when compared with MCAO+CORN group, #*P* <0.0001,**P<0.001 | | | | | | | |

It was found that no significant difference was observed between SHAM and MCAO groups in body weight before or after MCAO operation (Table 5.1). However, all the other parameters were significantly deteriorated in MCAO group compared with SHAM operation groups, including neurological deficit scores, infarction volume, BBB leakage, water content and MDA level (Table 5.1).

Taken together, the acute ischemic stroke model at 24 hours post onset of cerebral I/R, the brain damage was confirmed by increased neurological deficit scores, infarction volume, brain edema and BBB leakage in the MCAO groups (Table 5.1). It means our MCAO induction was success. In addition, the results were in line with Wang's model [28]. Therefore, this model could be able to use for the following experiment of curcumin study.

5.2 To study the effects of curcumin on I/R injury using MCAO-rat model

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In this part, the neuro-protective effect of curcumin was monitored. The results of the present study showed that curcumin exerts neuro-protective effects upon cerebral ischemia reperfusion damage, by preventing the deterioration of neurological dysfunction, infarction volume, brain edema and BBB disruption. The underlying mechanism might be associated with the down-regulation of MDA, ICAM-1, MMP-9, NF-κB and caspase-3, and the up-regulation of Nrf2 expression.

Curcumin as the main ingredient of curcuminoids, which belongs to polyphenol compounds in turmeric [157]. It has been proven that curcumin

possesses a broad spectrum of bioactivities, including anti-inflammation, anti-oxidation, anti-apoptosis, and neuro-protective properties [96]. In addition, it exhibits extremely safety for human, which has been confirmed by a phase I clinic trial with 12 g oral supplement in USA [158]. Another clinic trial found that curcumin oral administered with 8g/day for 3 month without any adverse effect [159]. However, its application has been restricted to its low bioavailability. With oral administration, the absorbed curcumin was subjected to quick reduction and conjugation. After 1 hour intervention, it could reach to a low peak serum concentration of free curcumin. In Pan's study, 100 mg/kg oral administration of curcumin in mice, only 0.22 μg/ml of free curcumin was detected at 1 hour post administration. However, following i.p. administration of curcumin at same dose (100 mg/kg), the peak serum concentration of free curcumin reach to 2.25 μg/mL at 15 min post injection. The brain distribution was 0.41μg/g detected by high-performance liquid chromatography (HPLC) [6]. Therefore, the blood absorption of curcumin is much more effective by i.p. administration than oral. Hence, i.p. injection of curcumin was chosen to use in our study.

In addition, 1 hour transient MCAO model was considered to induce minimal pathological changes *in vivo* [160]. So the injury of our model is relatively mild, and that could facilitate curcumin perform its protective effects easier, even without adjuvants.

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The strong polar solvent is essential for curcumin dissolution due to its hydrophobic nature, but lipid soluble characteristics. DMSO and NaOH are commonly used solvents for curcumin. However the toxic effect has been reported. DMSO could cause retinal apoptosis *in vivo* with low dose of i.p. injection (5 μl). In addition the neuronal apoptosis was also observed with 2-4% low dose of administration [71]. Moreover, developmental neurotoxin was also reported by Hanslick et al [161]. The NaOH is strong basic chemical could trigger internal environmental disturbance. Corn oil was used for our research to dissolve the curcumin. It has been used as solvent to carry medicines in accumulating researches [162-165]. However, to our knowledge, there are very few works used corn oil as solvent for MCAO model.

Based on many studies, curcumin have accumulating neuro-protective effects against stroke [166][167]. Its protective effects mainly depended on the prerequisite condition to pass through the BBB. For our understanding, the ability of curcumin to pass through the BBB could be partially attributed to the following reasons,

i) The hydrophobic property facilitate curcumin to penetrate the BBB [168].

ii) The blood absorption and brain distribution of curcumin was improved by i.p. when compared with oral administration [6], as mention above.

iii) The disrupted BBB caused by MCAO make curcumin easier to pass.

BBB disruption upon the cerebral I/R could be roughly differentiated into 2 stages. The first stage of BBB leakage normally starts from 3 hours after reperfusion.

And this is relatively reversible damage stage compare with the later one. It is also promising phase to retrieval the function of BBB. The later stage of BBB leakage, which is start from 18 to 96 hours post reperfusion, and normally regarded as irreversible stage [62]. This temporal characteristic effect is partially overlapped with pharmacokinetic property of curcumin.

Even though most of the report found that the metabolic rate of curcumin was quite fast by using HPLC assay. The peak serum concentration of curcumin was normal at 1 hour post administration. However, it was declined fast within 1 hour [6]. But using [$^{14}$C]curcumin, Perkins found that in mice brain tissues, the level of curcumin was detected start from 1 hour to 24 hours (i.p. 100mg/kg) within 10-20% peak value [169]. Hence, it could be predicted that increased BBB leakage could facilitate more curcumin to pass through the BBB. In addition, the most important evidences came from the preliminary studies, that i.p. administration of curcumin at 30 min after cerebral ischemic injury could attenuate brain injury in dose-dependent manner rat model (1 hour cerebral ischemia and 1 hour recirculation) [170, 171]. Thereby it might be the circumstantial evidence to demonstrate the ability of curcumin pass through BBB to perform its neuro-protective effects.

5.2.1 Effects of curcumin on MCAO-induced BBB leakage

Our results demonstrated that the BBB leakage in MCAO+CUR was significantly decreased compared with MCAO+CORN group (Figure 4.3). As it is well

known upon I/R injury, the BBB impairment occurs in short period of hyperemia (mins) followed by two hypo-perfusion episodes (no flow). The former episode will happen at 3-8 hours after reperfusion. The Later phase initiates from 18 hours to 96 hours post reperfusion [51, 61]. Evans blue dye is the most common stains for BBB integrity assay [172-174]. Whereas, this agent could inhibit the glutamate uptake [175, 176], a key factor contribute to BBB disruption [177].

In addition, the intra-vital observation could provide images of single pial vessels in real time manner [94]. Therefore the intra-vital observation of BBB disruption by confocal microscopy was chosen for our study. So far very few studies reported the BBB integrity by checking FITC-leakage via intra-vital microscopy [95, 178]. Among them the less researches was related to stroke [179]. Most of intra-vital microscopic observation focused on the leukocytes infiltration upon stroke [40, 180, 181], or aimed at early stage of BBB disruption (within 3-4 hours of stroke onset) [179]. Regarding to the later episode, that is irreversible stage compared with former one, and more contributes to the life-threatening consequence, edema and hemorrhage [62].

The one research observed that it was significantly increased FITC-dextran-2000 kD extravasation at 30 min of injection from the pial vasculature in stroke mice (30min MCAO and 24 hours reperfusion model) [182]. To our knowledge, our study might be the first time to detect BBB opening by intra-vital microscopy at 24 h post reperfusion in rat stroke model. However, in our study, the FITC-dextran-250 kD was

used instead of 2000 kD. Due to the bigger molecular weight is much harder for BBB penetration. Therefore, the BBB leakage (10min post injection) occurred earlier than Jin's study (30 min post injection) [182]. In addition, our result was similar as the reported by Eunice's research [183]. They found that slow leakage was occurred at around 5-15 min post injection in ultrasound-induced BBB opening. Furthermore, Kureger also reported that the BBB disruption was increased at 25 hours after embolism stroke model induction through FITC-immunohistochemistry detection compared with SHAM operation group [184].

In our study, as for the location of leakage, started from post-capillary venules with around 20-40 μm in diameter, and it was consistent with Cho's research [183]. The reason could be attributed to the common event appeared for inflammatory/immunological responsibility that mostly resigns on venule-site. Together with low shear stress, post-capillary venules tend to be vulnerable site for BBB integrity upon the inflammation and prone to leukocyte-endothelial cells interaction [185].

The neuro-protective effect of curcumin on the BBB integrity upon cerebral I/R injury, has been confirmed by Evans blue test with curcumin i.v. injection at 30 min [166]. The reason could be implicated to enhancing expression of HO-1 [186]. Whereas, the data of intra-vital microscopic observation is not available at 24 hours after reperfusion. Our results demonstrated that curcumin pre-reperfusion intervention could partially prevented the BBB leakage compared with MCAO+ CORN

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group (Figure 4.3). Which could be a useful data to compensate the loss of intra-vital observation on BBB.

5.2.2 Effects of curcumin on MCAO-induced brain edema

As shown in Figure 4.6, the inhibition of edema, was exactly observed in MCAO+CUR group. Edema is one of the consequences of BBB opening, could trigger herniation to threaten patients' life. 24 hours after I/R, the brain edema results from the vasogenic water translocation [65]. In our study, the brain edema was detected by measuring the brain weight between fresh and dry tissue. The preliminary studies found that the brain water content in transient MCAO group was significantly higher than SHAM operation group [23, 187]. And percentage of water content in MCAO+CORN groups (Figure 4.6) was in line with Sun's report in rat model (1h MCAO and 24 h reperfusion) [156]. The edema was associated with abnormal aquaporins (AQP) expression, which are water pore-forming proteins that make water pass through BBB.

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In our study curcumin administration partially prevented edema against cerebral I/R injury, which was consistent with Thiyagarajan's research in transient MCAO model [167]. Due to 24 hours post reperfusion, the edema mostly is vessel-originated, which is related to the BBB disruption. Therefore the inhibition of BBB leakage might decrease edema, which was exactly observed in our correlation results

(Figure 4.12). The prevention of edema was concurrent with attenuated BBB disruption upon curcumin intervention in transient MCAO model.

5.2.3 Effects of curcumin on MCAO-induced infarction

By using 2% of TTC staining detection, the infarction volume of MCAO+CUR was significantly decreased compared to MCAO+CORN group (Figure 4.2). The 2% of TTC staining is widely accepted method to assess lesion size in rat brain post cerebral I/R injury [135]. TTC is the substrate of cellular dehydrogenases that existed in mitochondria. In viable tissue, it will be reduced to formazan which stained the area with red color. However in dead tissue there will be no color developed (white color) [188]. As our study found that the infarct volume detected (Figure 4.2) by TTC staining was 32.96 ± 1.871 and 26.91 ± 1.563 for MCAO+CORN and MCAO+CUR groups. These results were slightly higher than preliminary reports by Wang and Kang research group [28]. But consistently with reported by Wang-Fisher [129] and Zhao [45]. The reason could be the monofilament suture was from different company, and the silicon-coated technique was different, which could directly influence the outcome and variability of model induction [129]. Regarding to the location of infarction areas, it was in line with previous study, which located on the lateral cortex and sub-cortex areas [28]. As for the effect of curcumin on the infarction volume, significantly decreased infarction volume was found in MCAO+CUR group compared with MCAO+CORN group (Figure 4.2). This result was concurred with

reduced neurological deficit scores (Figure 4.13) by curcumin administration compared with corn oil treatment. It was in line with previous studies [40, 124, 189-191].

Regarding to the mechanism of neuro-protective effects of curcumin against cerebral I/R injury, it could be attributed to its wide spectrum of biological and pharmacological activities, including antioxidant, anti-inflammatory, and anti-apoptosis properties that will be discussed more in the following session.

5.2.4 Effects of curcumin on MCAO-induced oxidative stress

From our findings, the increased oxidative stress could significantly decreased in MCAO+CUR when compared to MCAO+CORN group as represented by the levels of MDA shown in Figure 4.5. As mentioned in literature review, oxidative stress is one of the primary factors contributed to the cerebral I/R. Besides the indirect damage effect on the brain tissue, it could directly attack the protein, nucleic acid and lipid. For protein, oxidative stress could modify the molecular structure of protein to become fragmentation and proteolysis [192]. Regarding to the nucleic acid, the oxidative stress may directly attack either sugar or DNA molecules [193]. For the lipid, the reactive oxygen species work on the fatty acid composition to modify the polyunsaturated fatty acids in a self-propagating chain reaction way. The modified membrane lipids are particularly harmful to the viability of cells, even tissues [194]. The direct measurement of free radicals is quite difficult task [195]. Therefore

significant elevated MDA level (lipoperoxidation biomarker) or decreased of anti-oxidation agents in the stroke patients [196, 197] and animal models [156, 198] are mostly performed.

As described in previous studies, the MDA level was assayed because MDA is the oxidized product of lipid molecules that will increase as oxidative stress occurred. The level of MDA in corn oil treated MCAO group (1.173 ± 0.09964, #$P$<0.0001) was significant increased as compared to SHAM group as shown in Figure 4.5. This result was in line with the Sun's report [156]. It confirmed that, in 1 hour transient MCAO rat model, the oxidative stress was elevated compared with its counterparts at 24 hour post reperfusion. However, in our results, the MDA level in MCAO group was around 100% higher than SHAM group. In Sun's report, the increased MDA level in stroke model group was about 57% higher than SHAM [156]. The reason could be attributed to the MDA detection kit, which was different from two research groups.

In our study, the increased oxidative stress was found by over-production of MDA level in corn oil treated MCAO group. However, curcumin could dramatically inhibit the increased of MDA level as shown in Figure 4.5. It was consistent with Ghoneim's research, which was the first work to illustrate the effects of curcumin on cerebral I/R injury through anti-oxidation property [170]. In his study, besides MDA level, the production of XO and $O_2^-$ were also decreased in transient MCAO rat model with curcumin intervention at 30 min after ischemia. The time of curcumin

administration was similar to our procedure. Thiyagarajan study group also found 300 mg/kg intraperitoneal injection of curcumin could inhibit free radical to perform neuroprotective effects on stroke [167].

The mechanism of anti-oxidative stress of curcumin was ascribed to its molecular structure, which contains phenolic group that could normalize free radical molecule to stable molecule by donating H-atom [118]. In addition, curcumin could enhance the transcription factor of Nrf2 to increase the production of antioxidant enzymes, superoxide dismutase, catalase, and glutathione peroxidase [199]. It was also confirmed *in vivo* and *in vitro* studies done by Wu and his co-workers [200]. The neuro-protective effect of curcumin was involved in the activation of Akt/Nrf2 pathway [200].

Recently Nrf2 has been regarded as potential therapeutic target for stroke. That is a transcription factor constitutively expressed in the cytoplasm. Upon the activation by oxidation, which could translocate into the nucleus to mediate transcription of endogenous protective enzymes against oxidative stress. This includes superoxide dismutase (SOD), glutathione peroxidase, catalase, thioredoxin, peroxireoxins and heme oxygenases [88, 93].

Preliminary studies have reported that elevating Nrf2 activity could induce protective effects on stroke models [93, 200, 201]. Yang et al. found that by using immunohistochemistry assay, upon permanent MCAO injury, the positive Nrf2 staining cells number was significantly increased in ischemic cortex area, starting from 3 hours

to 72 hours post injury, with peak time at 24 hours [92]. For 1h transient MCAO mice model, the expression of Nrf2 was also found increased from 2 hour to 72 hours after reperfusion, peaking at 8 hours [202]. Therefore, in our present study, the Nrf2 expression was detected by immunohistochemistry at 24 hours post reperfusion.

Even though there was no significant difference in total Nrf2 positive staining cells number between injury and non-injury side of corn oil treated MCAO group. The trend of higher Nrf2 positive staining cells number in injury hemisphere was observed in cortex area (Figure 4.7). Normally, to maintain the homeostasis of body, when the power of oxidative stress increased, activation of Nrf2 will be enhanced as one of anti-oxidative stress ability [88, 93]. That is the reason why the increased level of Nrf2 was observed in injury side of MCAO group compared with non-injury side. However, the substantial oxidative stress either from NF-κB activated inflammation or oxygen burst of reperfusion was too vigorous. Which offset the increased anti-oxidant capacity from Nrf2 activation. Therefore, the slightly enhanced level of Nrf2 expression observed in the injury side of corn oil-treated MCAO group was not significant *vs.* non-affected side. These results were similar as research of Soejima, there were no significant difference of Nrf2 protein expression in both nucleus and cytoplasm between non-injury and ischemic side by Western blot [203].

Curcumin is a potent anti-oxidative agents, and its anti-oxidant property was reported 10 times stronger than vitamin E [204]. One of its anti-oxidative effect is performed through enhancing Nrf2 activation [92, 115, 205]. However, the effect of

curcumin on transient MCAO model still remained unclear, especially on Nrf2 expression. As for proximal MCAO animal model, the reports have found the severity of striatum area is comparatively stronger than surrounding cortex area. So striatum is more like as core area of death tissue, and cortex is more like penumbra area [153, 206]. As previous study found that, Nrf2 was boosted in penumbra area, but not core area in 24 hours MCAO rat model by immunohistochemistry [149]. Therefore the cortex area is what we focus in this study.

Simultaneously, the total Nrf2 expression by IHC was markedly higher in curcumin treated MCAO group than corn oil treatment as shown in Figure 4.7. This result could be the evidence to partially illustrate why the MDA level was lower upon the curcumin intervention. We could infer that, the increased endogenous defense enzymes via activation of Nrf2 after curcumin intervention, partially offset the deterioration effect from oxidative stress. Which was confirmed by Yang's study, that the HO-1 expression (endogenous anti-oxidative enzyme) was enhanced later than the activation of Nrf2 after the curcumin administration to permanent MCAO model [92]. Therefore, the neuro-protective effect of curcumin might through up-regulation of the Nrf2 to strengthen the potential of endogenous anti-oxidation for MCAO model. Which is related with decreased damage to the infarction volume, BBB leakage, edema and neurological dysfunction. As demonstrated by abundant reports [93, 200, 201].

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In addition, even though the damage severity of cortex is relatively milder than striatum, just take the cortex area into consideration, the comparative penumbra and core areas still could be differentiated by H&E detection (Figure 4.6). So in our IHC assay, we observed the Nrf2 expression in penumbra (A, D areas, border areas) and core area (B, C areas, central areas) (see Appendix A). However, our results didn't find any significant difference on these two areas. But the Nrf2 expression within the whole cortex area was significantly increased by curcumin intervention, which was consistent with the results of Shih [93].

Preliminary researches supported that nucleus Nrf2 expression was significantly increased upon the 1 hour transient MCAO model in the microglia, astrocytes, and neurons cells by double fluorescence detection [149]. The Nrf2 activation in the endothelial cell was also found upon subarachnoid hemorrhage rats model, and the concentration of nucleus Nrf2 was significantly increased compared with control group by electrophoretic mobility shift assay (EMSA) and western blot [207]. As for our results, no significant difference of Nrf2 was found, in the nucleus expression among different group and areas. The reason could be that the antibody of Nrf2 was not target on the activated form, so the significant expression could not be detected in the nucleus according to the antibody datasheet (Cat: ab31163, Abcam, UK). However, the results may be confirmed in our future study by using different methods including real-time PCR and ELISA.

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The mechanism behind the effects of curcumin on the Nrf2 might be addressed by the alteration of cysteine residues of Keap1, which activates the Nrf2-Keap1 conformational change and promotes the translocation of Nrf2 into the nucleus. As it has been reported that the translocation of Nrf2 into the nucleus can further induce the transcription and expression of ARE contained phase II enzymes [208].

5.2.5 Effects of curcumin on MCAO-induced inflammation: ICAM-1, MMP-9, and NF-kB

Inflammation is the other major factor that contributes to the brain injury upon cerebral I/R. Mainly they associated with inflammatory mediators and inflammatory cells infiltration. The induction of inflammatory cytokines and adhesion molecules were initiated immediately after onset of cerebral ischemia. For example, TNF-$\alpha$ and IL-1$\beta$ were found to be up-regulated by neurons in ischemic brain area [73], and detected in the cerebrospinal fluid taken from both stroke patients [209] and animal cerebral ischemic models [210]. Which was identified to related with enhanced para-cellular permeability of endothelial cells [211], cellular apoptosis and severity of ischemic injury [211].

Cytokines could trigger over-synthesis of chemokines, which play role to attract leukocytes invasion into the parenchyma upon a variety of brain injury [61]. To mediate the inflammatory cells transmigration, the firm interaction between endothelial cells and leukocytes by ICAM-1, plays essential role in BBB disruption

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[185], and obstruction of microvasculature ("no-flow" phenomenon) especially post reperfusion [212].

Our study has shown that the ICAM-1 expression appeared along the microcirculation system, and it was significantly higher in injury cortex when compared with non-injury side of MCAO+CORN group at 24 hours post-reperfusion (Figure 4.10). ICAM-1 is belong to the immunoglobulin family of cell surface proteins [51]. It is constitutively expressed on many cells, such as endothelial cells, fibroblasts, macrophages and activated lymphocytes [213] with low level under quiescent condition. Accumulating evidences have proved ICAM-1 overexpression in stroke patients [151, 182], and *in vivo* transient rats stroke model [152]. Zhang and Wang reported that after the cerebral ischemia, the ICAM-1 expression was increased, and reached the peak within 12–24 h after stroke onset, thus followed by the enhanced leukocytes transmigration [63, 64]. The clinic study also found that patients had more ICAM-1 expression in the infarcted zone than non-injury area [214]. In addition, inhibition of ICAM-1 dramatically decreased leukocytes adhesion and alleviated brain damage in transient stroke model [215-217]. The protective effect against stroke also obtained in ICAM-1 gene deficit mouse [154]. In our study, the elevated ICAM-1 level was paralleled with the increased BBB leakage and edema in MCAO groups (Figure 4.14). Which was coincidence with Liu's study [218]. Therefore, the treatment target on the ICAM-1 might bring beneficial effect on the cerebral I/R injury.

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Curcumin could exert its anti-inflammation property through inhibition of ICAM-1 expression in cancer [187] and diabetes [219]. However, upon the cerebral I/R injury, the report involved in the effect of curcumin on ICAM-1 was very rare. Recently, by using PCR, it indicated that 1-hour pre-reperfusion of curcumin could suppress the gene expression of ICAM-1. This correlated with dramatic inhibition of neutrophils adhesion in the injury brain area (4 hour ischemia plus 4 hours reperfusion rat model) [40]. In addition by using *in vitro* experiment, the inhibition effect of curcumin on ICAM-1 gene expression upon TNF-$\alpha$ stimulation was also demonstrated in HBMVECs [40].

By using immunohistochemistry, our data indicated that 24 hours post-reperfusion, the level of ICAM-1 in MCAO+CUR was significantly lower when compared to the MCAO+CORN group (Figure 4.8). The inhibited ICAM-1 expression was found in the whole cortex, penumbra and core areas (Figure 4.8). In addition, the decreased level of ICAM-1 was related to the alleviated brain edema, and BBB leakage by curcumin compared with vehicle treated MCAO group (Figure 4.15 and Figure 4.14). Therefore, the anti-inflammation effect of curcumin on the acute cerebral I/R damage could be presumed partially attributed by its action on ICAM-1 inhibition, as then further prevented the leukocytes adhesion and infiltration.

Concurrent with expression of inflammatory mediators and inflammatory cells infiltration, the excessive iNOS, free radicals, MMPs and other mediators are produced, especially post reperfusion [61]. Among them, MMPs are the critical

extracellular endopeptidases, can digest extracellular proteins (e.g. collagen, proteoglycans, elastin or fibronection) [220]. MMP-9 belongs to gelatinases, could activate pro-inflammatory agents, e.g. chemokines (CXCL-8) or cytokines (TNF-$\alpha$), trigger apoptosis to neurons [221]. However, the more important role of MMP-9 is to digest collagen type IV (basal laminal) and tight junction, both of them are main components of BBB, which contribute to the irreversible BBB disruption and vasogenic edema upon cerebral I/R [222]. Therefore, the activation of MMP-9 could facilitate the inflammatory cells break the integrity of BBB. As described in previous research, the increased risk of intracranial hemorrhage was positively related with the serum activated MMP-9 level [223]. This could predict the risk of hemorrhage transformation within 3 hours of stroke onset in thrombolysis treated patients [220].

In our study, Figure 4.16 showed that increased of the BBB leakage might associate with the level of MMP-9, which significantly enhanced in cortex area of affected hemisphere in MCAO+CORN group compared with contralateral side. This was consistent with Bauer’s study, which showed by gel zymography, the increased MMP-9 level was associated with elevated BBB leakage in the hypoxia brain area, and the increased BBB permeability was partially prevented by MMP-9 inhibition [224]. As previous research reported that the peak level of MMP-9 was at 24 hours post injury in both patients [225] and rat stroke model [226], it could be an indicator for the infarction volume, stroke severity and functional outcome [220]. However, our data did not show the correlation between MMP-9 expression with those parameters.

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For the location of activated MMP-9, the significant difference was identified in the cortex areas between ipsilateral and contralateral side for MCAO+CORN group. As many researchers reported the elevated MMP-9 expression was observed in both core and penumbra areas by gelatin zymography and western blot in 2 hours transient non-human primates [227] and patients [110].

The MMP-9 is excretive protein in the form of zymogen under normal condition. Upon the stimuli, it could be activated to degrade extracellular matrix of BBB. Therefore, activated MMP-9 normally expressed in extracellular space. On 24 hours post reperfusion, the cerebral endothelial cells and infiltrating leukocytes are regarded as the major cellular sources for MMP9 inducer [228-230]. So the MMP-9 is expected to secrete from infiltrated leukocytes and endothelial cells surrounding the microvascular systems. Our results were in line with Bauer's study [224] and our hypothesis, the increased MMP9 level was identified in the extracellular area near the microvasculature (Figure 4.9), where the elevated ICAM-1 was expressed (Figure 4.8). These data might be an indirect evidence to show the relationship among MMP-9, leukocytes, and BBB disruption in the cortex area (Figure 4.16). For non-injury side, spares expression of MMP-9 was found in the cytoplasm of cells, but no obvious expression was located on the blood vessels area, which was agreed with Rosell's study [110].

To our knowledge, most of researches just reported the curcumin effects on MMP-9 expression in cancer or diabetes mellitus related diseases [194, 195].

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Lately, *in vitro* study reported that curcumin inhibited lipopolysaccharide (LPS) stimulated astrocytes, which is related to reduction of MMP-9 expression level [196]. In 2013, Kelly research group found that right after reperfusion, curcumin could attenuate brain edema, neurological function which related to the decreased MMP-9 expression in transient MCAO rat model [189]. Those results were consistent with the present study. Except in Kelly's study, no significant effect on infarction volume was observed [189]. This little discrepancy could be attributed to the injection dose of curcumin (in present study =300mg/kg *vs.* their study=250 mg/kg), and the different experimental protocol (in present study 1-hour ischemia + 24 hours reperfusion *vs.* their study=3 hours ischemia + 21 hours reperfusion).

As shown in Figure 4.10, our results indicated that the cells number of NF-κB nucleus positive staining was strongly inhibited by curcumin treatment compared with MCAO+CORN group in both cortex and penumbra areas. It is quite commonly known that NF-κB plays essential role to regulate the body homeostasis under physiological and pathological circumstances. The activation of NF-κB pathway plays important roles in the oxidative stress, neuro-inflammation and cell survival related genes transcription [198]. Therefore, it may say that NF-κB activation is one of major mediators of inflammation. More than hundreds of inflammatory related proteins are regulated by NF-κB, including TNF, IL-1α and β, IL-6, iNOS, ICAM-1, and MMP-9 [44, 112]. In our study, the result of MCAO+CORN group showed that, the increased p65

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of NF-κB was significantly identified in the cell nucleus of ischemia area by immunohistochemistry (Figure 4.10).

The mechanism of NF-κB activation is attributed to the enhanced activity of IKK, which was found initiated ahead of the activation of NF-κB, as early as half an hour after stroke onset [231].

On the other hand, NF-κB activation is associated with cell survival. Hence the pro and anti-apoptosis function are both existed by NF-κB activation [14]. Upon ischemic stroke, the NF-κB activation mainly occurred in neurons, which normally contributes to neuronal cell death [14]. Besides neurons, the activation of NF-κB also observed in endothelial cells, astrocytes, and microglia upon stroke [232, 233]. However, the activation of NF-κB in different cells, the outcome varied. As report found that inhibition of NF-κB in neuron cells could reduce infarct size and the number of death cells, which was detected by TUNEL assay (dUTP-biotin nick-end labeling) upon MCAO injury [234]. However, there was no significant difference was obtained by inhibition of NF-κB in astrocytes [234]. By using immunohistochemistry, we showed that p65 was located in the nucleus at 24 hours reperfusion in MCAO rat model. Which suggested that NF-κB was activated and it may act detrimental effect upon MCAO injury in neurons. This could be result from the synchronously increased caspase-3 and infarction volume, which also observed in MCAO+CORN group.

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Among NF-κB family, 5 subunits are included, p65 and p50 take main deteriorated role in cerebral ischemia [14]. Our results showed elevated nuclear p65 expression in a transient MCAO rat model [235]. Which was in line with Nurmi's study, the nuclear translocation of p65 was demonstrated in biopsy brain tissue samples from human stroke patients [236]. In 2002, Xu et al observed improved infarction volume and neurological dysfunction in IKK gene mutant mice (2 hour ischemia and 24 hours reperfusion model) [237].

Regarding to the dual functions of NF-κB on the brain cell death, first, the explanation may be attributed to the severity of damage to the brain, upon not severe ischemic insults, NF-κB activation might prevent the brain cells death from the injury. However, upon long-lasting or strong damage, the protective property, normally related to the expression of downstream anti-inflammation protein, was offset by the over-production of pro-inflammatory proteins induced by NF-κB activation. Therefore, the NF-κB activation on the brain cells death might be varied in different model induction. Second, the results of the NF-κB activation might alter based on the assay method. Some studies detected the NF-κB by using EMSA, which just could check the gene expression other than the protein expression. Therefore, the increased expression of the NF-κB by EMSA could not illustrate the high expression of protein level. Third, the target cell of research might influence the result, due to the activation of NF-κB in different cells varied, e.g. neurons and

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astrocytes [234]. Thus, simply perform research with isolated culture cells couldn't completely imitate the complex internal environment of body. Therefore, *in vivo* and clinical studies might provide more accuracy evidence to check the effect of NF-κB activation upon stroke in systematic way.

It has been demonstrated that the strong inhibition property of curcumin on NF-κB activation is through suppression of IKK activity, IκB phosphorylation and NF-κB nuclear translocation [238] against cancer [239], diabetes [240], and cardiovascular disease [241].

However, it has also been suggested that the anti-inflammatory effect of curcumin is conveyed through the inhibition of AP-1, not NF-κB in a brain damage model induced by dopamine injection [242]. Few studies have reported the down-regulation effect of curcumin on NF-κB in brain diseases associated with traumatic models *in vitro* [243] and alcohol-induced brain impairment [244]. Therefore, not too many studies reported the effect of curcumin on NF-κB activation upon cerebral I/R injury. Lately some studies have reported the protective effect of curcumin on the brain injury upon stroke. The inhibitory effects of curcumin on NF-κB activation and subsequent ICAM-1 gene expression have been demonstrated in TNF-α-stimulated HBMVECs *in vitro* [40]. In 2014, Tu's research group found that two dose of curcumin administration attenuated infarction volume, neurological dysfunction and brain

edema in permanent cerebral ischemia rat model, which was related with the down-regulation of NF-κB activation [245].

Our results showed that the level of NF-κB in MCAO+CUR group was almost half amount of the MCAO+CORN group in penumbra areas (Figure 4.10). Moreover, there was no significant effect of curcumin in core area during cerebral I/R injury (Figure 4.10). This results demonstrated that curcumin could have its neuro-protective effect on penumbra area than core area compared with core oil treatment. Because during the cerebral I/R injury, the cells in penumbra undergoes milder damage than core area. In that case curcumin could prevent further damage to those cells via decreasing the expression of NF-KB, and then ultimately reducing the cell inflammation.

The inhibitory effects of curcumin on the expressions of ICAM-1 and MMP-9, could benefit up to 50% reduction when compared to MCAO+CORN group as well.

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The results may indicate the correlation between the NF-κB and inflammatory mediators, e.g. ICAM-1 (Figure 4.18) and MMP-9 (Figure 4.19). Therefore, our results could be used as a strong support for anti-inflammatory effect of curcumin through the evidence of NF-κB cascade inhibition, particular by using *in vivo* stroke model.

5.2.6 Effects of curcumin on MCAO-induced brain cell death

Interestingly, our results showed that the number of positive nucleus caspase-3 was significantly decreased in MCAO+CUR when compared to MCAO group

in penumbra area (Figure 4.11). It is believed that the mutual efforts from oxidative stress and inflammation are the underlining causes of brain cell death. Cell death could be divided into necrosis and apoptosis which depends on the severity and duration of damage to cell. For the ischemic stroke, necrosis normally occurs in gusty, permanent vascular blockage, whereas, reversible ischemia with reperfusion tends to milder damage, apoptosis are more prominent, especially within ischemic penumbra area [246]. Furthermore, for the same type of stroke, the type of brain death is varied as well. Right after ischemic stroke, the apoptosis was observed in the core area. As the duration and severity of ischemia is prolonged and augment, eventually necrosis is prevail on core area. Regarding to the peri-infarction area, due to the collateral circulation, the energy supply is partially impaired, but not blocked. Therefore, the apoptosis is more prominent, especially mitochondrial involved apoptosis, which is more energy-dependent process [247].

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Caspase family as an executer plays key role to trigger mitochondrial involved apoptosis. It is cysteine-dependent aspartate-directed proteases (Friedlander, 2003). Studies have found penumbra neuronal apoptosis is closely related with caspase-3 activation following cerebral I/R [72, 248, 249]. Apoptosis increases at around 6-10 h in penumbra area and peaks at 24-48 h after reperfusion [250, 251], which is consistence with elevated caspase-3 level [249]. The inhibition of caspase-3 has been proven a promising way to reduce infarction volume and restore

neuronal deficit function upon cerebral I/R damage [252]. In present study as shown in Figure 4.11, for penumbra area, the cells number of nucleus caspase-3 significantly enhanced in injury side compared with non-injury side upon 24 hours cerebral I/R injury. Which was in agree with the elevated infarction volume for the MCAO groups (Figure 4.20). It was concurrent with the research of Graham and Zhao [45, 253].

The enhanced caspase-3 level was markedly expressed in penumbra and whole cortex areas of injury side compared with non-injury side. These results were consistent with Manabat's study, in that study, they found the activated caspase-3 was gradually increased following duration of reperfusion until 24 hours by western blot. But more prominent caspase-3 level was observed in core area [254]. The discrepancy about the caspase-3 distribution on the penumbra and core areas could be aroused from the different methodology. Because in Cheng's study, enhanced activated caspase-3 was localized in neurons of penumbra area, which was detected by NeuN labeled immunohistochemistry. It was in line with increased of apoptotic morphological TUNEL positive cells [130]. The positive cleaved caspase-3 cells in penumbra was also reported by Zhang's research group [255]. Our study also in line with those findings. However, in our study, we also found that during the curcumin treatment there were significant inhibition of caspase-3 activity in penumbra area than core area (Figure 4.11). As known that during the cerebral I/R injury, core area had more severe damage than the penumbra area, by this fact it explains our findings that there was no significant difference in caspase level between

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MCAO+CORN and MCAO+CUR groups. In addition, based on this idea, it is reasonable to support our result that curcumin could significantly prevent further cell death in penumbra area, where most cells still remain alive.

In addition, the elevated infarction volume and deteriorated neurological dysfunction was synergistically presented in MCAO+CORN group. Which is in line with Zhang's study, the increased infarction volume and neurological deficit was observed in transient MCAO group [255]. Therefore, it could be an evidence to confirm the increased caspase-3 related to the increased cell loss and infarction volume (Figure 4.20), which contribute to augment of neurological dysfunction (Figure 4.22). Which was in line with previous studies [69, 246, 253, 256].

Cell death is end stage of cells upon cerebral I/R injury. It could be influenced by any stimuli mainly related to inflammation and oxidative stress. Therefore, any intervention target on the above factors might partially attenuate the brain cell death. Thanks to the multiple effects of curcumin on the inflammation and oxidation, which could partially blocked the upstream stimuli for cell death. Simultaneously, curcumin could regulate transcription factor to perform its neuro-protective effect. Many transcription factors had cross talk with apoptosis process. For instance, the activation of NF-κB might be either pro or anti apoptosis as discussed above, but activation of Nrf2 could alleviate apoptosis [257]. Therefore, the

adjustment of NF-κB and/or Nrf2 might alter the cell death process, including apoptosis.

In our study, the cells number of positive nucleus caspase-3 was significantly lower in curcumin treated MCAO group than vehicle treated MCAO group in penumbra area (Figure 4.11). The corresponding reduced infarction volume, BBB leakage, edema and neurological dysfunction were obtained in curcumin treated MCAO group compared with corn oil treatment. These results might provide evidence that reduced infarction volume (Figure 4.20), BBB leakage (Figure 4.23), edema (Figure 4.21), and attenuated neurological deficit (Figure 4.22) might be related to decreased caspase-3 by curcumin administration. Which was in line with Zhao's study [45].

Up to this point, it is worth to make the conclusion remark here that the mechanisms of curcumin on caspase-3 against cerebral I/R injury may be primarily contributed by the down-regulation of inflammation responses, and the inhibition of oxidative stress.

However, it is suggested that the further study should use the double immunofluorescence staining to investigate the cells location of each protein with related cell label biomarkers, eg. neuN (neuron), GFAP (astrocyte), or VE-cadherin (endothelial cell), and by using PCR to detect the gene expression of proteins, and qPCR or ELISA to accurately check the amount of protein expression.

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From the present data, it appears that curcumin could provide beneficial effect to prevent neurological dysfunction, infarction volume, BBB leakage and edema upon cerebral I/R injury, the underlying mechanism could implicate to down-regulation of MDA, ICAM-1, MMP-9, caspase-3 and NF-κB, and up-regulation of Nrf2 production. Therefore, the current results support our proposed hypothesis and the proposed mechanism of curcumin effect, which was summarized in Figure 5.1.

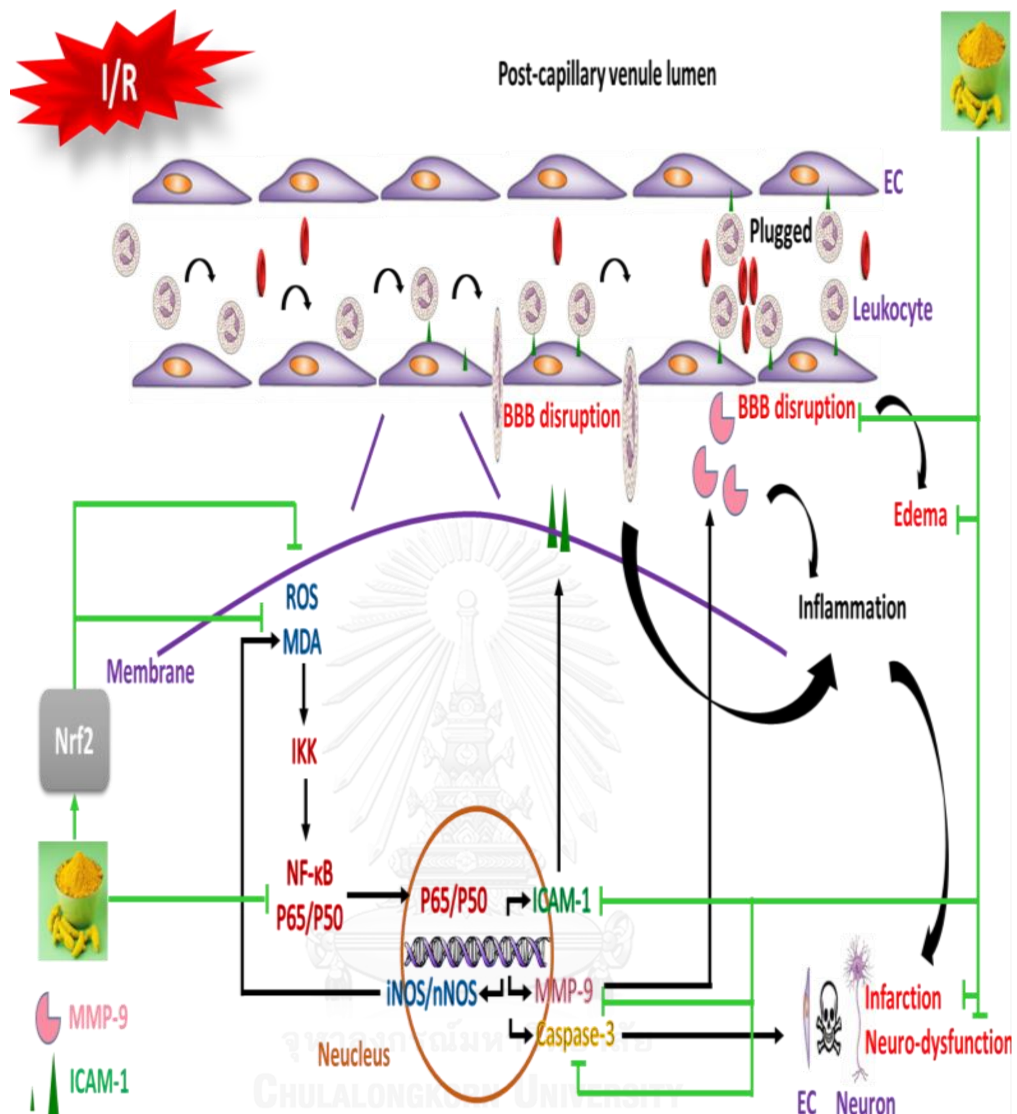


*Figure 5. 1 Proposed neuroprotective effects of curcumin on cerebral I/R injury. Referring back to the hypothesis of the present study, which is, "Curcumin can attenuate neuronal cell death and protect BBB integrity against ischemia/reperfusion injury, which is related to regulate expression of ICAM-1, MMP-9, caspase-3, NF-*$\kappa$*B and Nrf2 pathways in transient MCAO stroke rat model." The obtained results confirmed our hypothesis.*

# CHAPTER 6

## CONCLUSION

This study was designed to investigate the effects of curcumin on the MCAO rat model. To illustrate the mechanism through which curcumin perform its neuro-protective effect, Nrf2, ICAM-1, MMP-9, NF-$\kappa$B and caspase-3 expression were detected.

The main findings could be concluded as follows:

1. Curcumin (300 mg/kg, i.p.) attenuated neurological dysfunction when compared with vehicle treated MCAO group ($P<0.05$).
2. Curcumin could significantly decrease infarction volume in MCAO group rat ($P<0.05$).
3. BBB leakage could be prevented by curcumin administration in MCAO groups ($P<0.05$).
4. Brain water content was significantly reduced by curcumin in MCAO groups ($P<0.01$).
5. Curcumin significantly lower the level of MDA as compared with vehicle treated MCAO group ($P<0.05$).
6. The expression of Nrf2 was enhanced by curcumin in cortex area of MCAO group ($P<0.05$).

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7. Curcumin inhibited the expression of ICAM-1 in cortex, penumbra and core areas of MCAO group ($P<0.01$).
8. Curcumin suppressed MMP-9 expression in cortex area of MCAO group ($P<0.01$).
9. The activated NF-$\kappa$B expression was repressed by curcumin in cortex and penumbra areas of MCAO group ($P<0.05$ for cortex, $P<0.01$ for penumbra areas).
10. The nucleus expression of caspase-3 was reduced by curcumin in penumbra areas of MCAO group ($P<0.05$).

In conclusion, based on our investigation, upon cerebral ischemia reperfusion injury, pre-reperfusion of curcumin intervention could prevent the brain damage by reducing neurological deficit scores, BBB leakage, edema and infarction volume. Which could be related to its down-regulation effect on MDA, ICAM-1, MMP-9, caspase-3 and NF-$\kappa$B, and up-regulation on Nrf2 production. In addition, curcumin also prevent the further damage to the penumbra area where the cells is still viable, but dysfunction. This will be one of novel findings of our work, which could illustrate neuro-protective effect of curcumin. These results suggest that curcumin might be a feasible strategy for treating cerebral I/R injury in the future.

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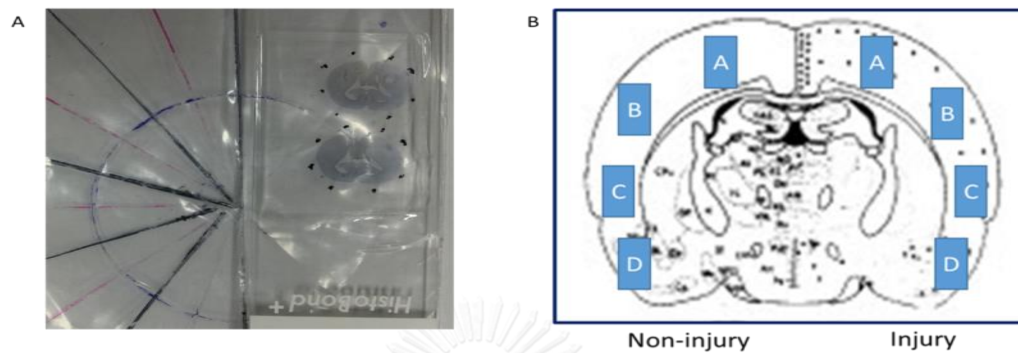
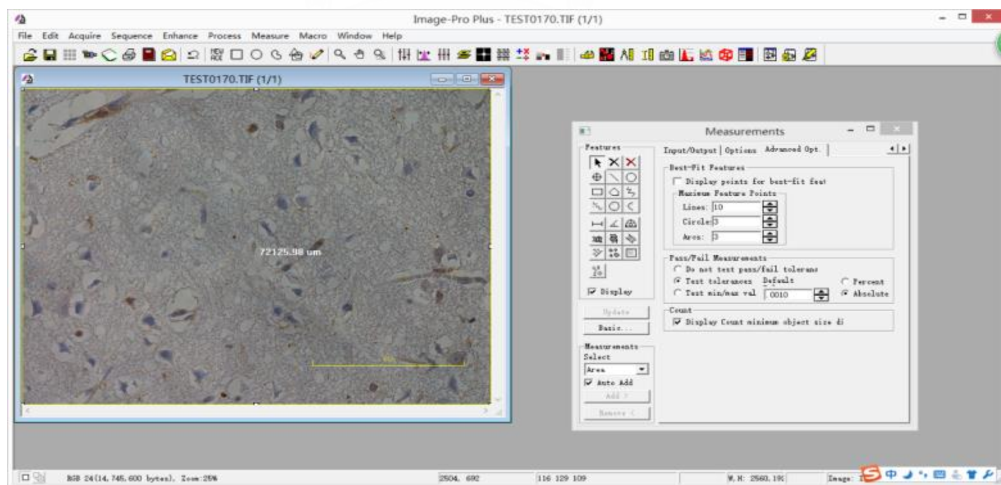
# APPENDIX




A. The penumbra and core area on IHC slice

*Figure A. The penumbra and core area of interest on IHC slice. With the tool of transplant radial map ruler (A), to locate four areas of interest (A-D) on the cortex of both non-injury and injury side. A, D mean penumbra areas; B, C mean core areas. The total results of A, B, C, and D represented the whole injury part of cortex area.*




B. The total size of IHC slice analyzed by Image-Pro plus 6.0

*Figure B. The total size of IHC slice by Image-Pro plus 6.0. The total size of each IHC slice was confirmed the same.*

# VITA

PERSONAL INFORMATION

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EDUCATION

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EXPERIENCE

Jul. 2001- Jul. 2011 Working as a doctor and a lecturer in the Second Affiliated Hospital of Kunming Medical University and Department of Laboratory Medicine, Kunming Medical University.

REWARDS

2011 Neighboring Countries Scholarship for Ph.D. Program from Chulalongkorn University

Chulalongkorn University

PRESENTATIONS

1 Curcumin protect BBB against ischemia/reperfusion injury related to regulate MMPs, ICAM-1 activation and decrease leukocytes adhesion in transient stroke rat model. International Workshop on Protein Expression and Purification Strategies, Oct. 28th - Nov. 1st, 2013.

2 The acute ischemic stroke rat model. 16th TSM Annual Meeting of Thai Society of Microcirculation, Dec.20th, 2013

3 The Neuro-protective Effects of Curcumin against I/R Injury in Transient MCAO Rats Model.43th Annual Scientific Meeting of The Physiological Society of Thailand, Apr. 23-25, 2014

4 Pre-reperfusion of curcumin could protect blood-brain barrier against I/R injury associated with Nr2, NF-kappa B, and caspase-3 expression in transient MCAO rat model. 10th World Congress for Microcirculation in Kyoto, Sep. 25-27, 2015