ELUCIDATING THE ROLE OF BAICALEIN-ENRICHED FRACTION TO MODULATE ISCHEMIC STROKE RECOVERY IN RAT MODEL

FARAH AMNA BINTI OTHMAN

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ELUCIDATING THE ROLE OF BAICALEIN-ENRICHED FRACTION TO MODULATE ISCHEMIC STROKE RECOVERY IN RAT MODELS

by

FARAH AMNA BINTI OTHMAN

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LIST OF SYMBOLS

<	less than
>	more than
-	minus
%	percentage
α	type I errors
β	type II errors
δ	effects size
σ	standard deviation
°C	degree celcius
Ca^{2+}	calcium
cm	centimeter
CO_2	carbon dioxide
C_T	threshold cycle
g	gram
H^+	protons
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
i.p	intraperitoneal
K^+	potassium
kg	kilogram
LD_{50}	lethal dose
Na^{2+}	sodium
mg	milligram

mL	milliliter
mm	milimeter
mM	micro molar
m/z	mass/charge
ng	nanogram
nm	nanometer
O_2^-	superoxide
$ONOO^-$	peroxynitrite
pH	potential hydrogen
psi	pound per square inch
R_f	retention factor
µg	microgram
µL	microliter
µm	micrometer
x g	relative centrifuge force

LIST OF ABBREVIATIONS

ALB	Albumin
ALP	Alkaline phosphatase
ALS	Amyotrophic lateral sclerosis
ALT	Alanine aminotransferase
AMI	Myocardial infarction
ANGPT1	Angiopoietin 1
ANOVA	Analysis of Variance
ARASC	Animal Research and Service Centre
AST	Aspartate aminotransferase
ASVCP	American Society for Veterinary Clinical Pathology
ATP	Adenosine triphosphate
BEF	Baicalein-enriched fraction
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factors
CO_2	Carbon dioxide
CBF	Cerebral blood flow
cDNA	Complementary deoxyribonucleic acid
CLSI	Clinical & Laboratory Standards Institute
CNS	Central nervous system
CoA	Coenzyme A
DG	Dentate gyrus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPX	Distyrene, Plasticizer and Xylene
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ESI	Electron Spray Ionization
ETC	Electron transport chain
ET-1	Endothelin-1
FDA	Food and Drug Administration
$FADH_2$	Flavin adenine dinucleotide

GABRA6	Gamma-aminobutyric acid type A receptor subunit alpha 6
GHS	Globally Harmonized System
Hb	Haemoglobin
H&E	Hematoxylin & eosin
HPRT1	Hypoxanthine phosphoribosyltransferase 1
IACUC	Institutional Care and Use Committee
JAKMIP1	Janus kinase and microtubule interacting protein 1
LC-MS	Liquid chromatography-mass spectroscopy
LD_{50}	Lethal dosage 50
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCP-1	Monocyte chemoattractant protein 1
MCV	Mean corpuscular volume
MIP-1	Macrophage inflammatory protein
MS	Multiple sclerosis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide powder
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen
Nfe2l2	NFE2 like bZIP transcription factor 2
NGF	Nerve growth factor
NMDAR	N-methy1-D-asapartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NFKB	Nuclear factor kappa B subunit 1
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NSCs	Neural stem cell
OECD	Organization for Economic Cooperation and Development
OGD	Glucose and oxygen deprivation
PBS	Phosphate buffered saline
PCV	Packed cell volume
RBC	Red blood cells
RMS	Rostral migratory stream
RNA	Ribonucleic acid
ROS	Reactive oxygen species

ROW	Relative organ weight
rpm	Revolutions per minute
RPL13A	Ribosomal protein L13A
rt-PA	Recombinant tissue plasminogen activator
SD	Sprague Dawley
SEM	Standard error of mean
SFM	Serum free medium
SGZ	Subgranular zone
SOD	Superoxide dismutase
SOD2	Superoxide dismutase 2
SPSS	Statistical package for the social sciences
SSF	Slow sand filter
STAT6	Signal transducer and activator of transcription 6
SVZ	Subventricular zone
TCA	Tricarboxylic acid
TIA	Transient ischemic attack
TLC	Thin layer chromatography
TP	Total protein
TTC	2, 3, 5-triphenyltetrazolium chloride
UPLC	Ultra-performance liquid chromatography
USM	Universiti Sains Malaysia
UV	Ultra violet
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A
WBC	White blood cells count
WHO	World Health Organization

LIST OF APPENDICES

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MENJELASKAN PERANAN FRAKSI YANG DIPERKAYA BAICALEIN UNTUK MEMODULASI PEMULIHAN STROK ISKEMIK DALAM MODEL TIKUS

ABSTRAK

Strok iskemia merupakan antara punca utama kematian dan kehilangan upaya jangka panjang dalam kalangan orang dewasa di seluruh dunia. Pada masa ini, pengaktif plasminogen tisu rekombinan (rTPA) adalah satu-satunya ubat yang diluluskan untuk terapi strok iskemia. Keadaan ini lebih teruk kerana ubat ini mempunyai tempoh berkesan yang singkat (~3-4.5 jam) dan menyebabkan kesan buruk jika diberikan di luar waktu ini. Oleh itu, terapi berasaskan tumbuhan ubatan dengan kesan sampingan yang rendah dan mudah didapati telah diperkenalkan sebagai alternatif yang meyakinkan untuk rawatan iskemik strok. Kajian ini menguji potensi terapeutik sebatian diperkaya baicalein (BEF), iaitu bahan yang boleh melindungi saraf yang diekstrak daripada daun tumbuhan ubatan yang dikenali sebagai *Oroxylum indicum*. Sebelum sebarang kajian *in vivo* menggunakan sebatian atau ekstrak tumbuhan dijalankan, adalah penting untuk memastikan profil keselamatan dan keberkesananya melalui ujian toksikologi yang menyeluruh. Dalam kajian ini, penilaian preklinikal akut dan neurotoksikologi subakut BEF telah dilakukan berdasarkan Garis Panduan 420 dan 424 yang telah ditetapkan oleh Organisasi untuk Kerjasama dan Pembangunan Ekonomi (OECD). Kajian ini mendapati dos yang boleh meyebabkan separuh kematian (LD_{50}) adalah lebih daripada 2000 mg/kg, tanpa kesan toksik berkaitan dengan rawatan atau gangguan neurotoksisiti, di samping tiada perubahan dalam penilaian hematologi, biokimia dan histopatologi dalam kedua-dua kajian neurotoksisiti akut dan neurotoksikologi subakut. Mengambil kira semua

keputusan yang diperolehi, adalah jelas BEF selamat untuk diambil secara oral dan berpotensi untuk dijadikan sebagai ubat oral untuk rawatan strok iskemia. Maka, sebanyak 50 mg/kg BEF diberikan secara oral kepada tikus Sprague Dawley (SD) (n=5) selama 4 hari sebelum induksi strok iskemia meggunakan endothelin 1 (ET-1). Kajian ini mendapati penggunaan BEF pra-induksi strok iskemia boleh memberi perlindungan yang ketara kepada tisu otak daripada kecederaan iskemia, seperti mana yang ditunjukkan oleh fungsi tingkah laku neurologi yang semakin baik, jumlah infark otak dan skor histologi melibatkan kemerosotan neuron yang jauh lebih rendah dalam kumpulan tikus yang dirawat dengan BEF, berbanding dengan yang tidak dirawat. Disamping itu, kajian ini juga menguji potensi BEF sebagai agen prarawat untuk memberikan perlindungan kepada stem sel neural (NSCs) terhadap keadaan iskemia sebelum memindahkannya ke dalam model tikus strok iskemia untuk rawatan. Keputusan menunjukkan bahawa tikus eksperimen yang dirawat dengan NSCs prarawatan dengan BEF pada dos 3.125 μg/mL selama 48 jam bukan sahaja menurunkan dengan ketara jumlah infark otak, kemerosotan neuron dan kemasukan sel radang, malah ia juga menaikkan dengan ketara jumlah salur darah dan fungsi tingkah laku neurologi sepantas 24 jam selepas rawatan, berbanding dengan kumpulan tikus NSCs yang tidak dirawat dan kumpulan kawalan yang tidak dirawat. Malah, jumlah ekspresi gen angiogeni (ANGPT1), anti-oksidan (SOD2), anti-keradangan (IL-1Rn) and neuroprotektif (JAKMIP1, STAT6, NGF, NFKβ) juga meningkat dengan ketara dalam tikus yang dirawat dengan NSCs prarawatan dengan BEF. Kesimpulannya, BEF adalah ubat berpotensi dengan kesan neuroprotektif dan prarawat yang boleh digunakan untuk meningkatkan rawatan klinikal bagi strok iskemia pada masa hadapan.

ELUCIDATING THE ROLE OF BAICALEIN-ENRICHED FRACTION TO MODULATE ISCHEMIC STROKE RECOVERY IN RAT MODEL

ABSTRACT

Ischemic stroke is presently the top two leading causes of mortality and long-term adult disability worldwide. Up to date, recombinant tissue plasminogen activator (rtPA) is the one and only approved drug for ischemic stroke therapy. The situation is even worse when this standard therapy has very narrow therapeutic window (~3-4.5 hours) and dentrimental side effects if administered beyond the golden hour. Therefore, natural medicinal plants-based therapy with low side effect and high bioavaibility has emerged as a promising alternative for ischemic stroke. This study assessed the therapeutic potential of baicalein-enriched fraction (BEF), a neuroprotective constituent extracted from the leaves of medicinal plant known as *Oroxylum indicum*. Prior to any *in vivo* study using natural botanical extracts or fractions, it is crucial to validate their safety profiles and efficacy through comprehensive toxicological assessments. In this study, the preclinical acute and subacute neurotoxicology assessment of BEF was evaluated based on the Guidelines 420 and 424 set in Organisation for Economic Co-operation and Development (OECD). It was found that the half lethal dose (LD_{50}) of BEF was more than 2000 mg/kg, with no treatment-related toxicity behaviour or neurotoxicity impairments, no alteration in haematological, biochemical and histopathological assessments in both acute and subacute neurotoxicity study. Taking all results together, it was clear that the BEF was safe to be consumed orally and had potential to be developed as an oral drug for ischemic stroke treatment. Thus, 50 mg/kg BEF was orally administered to Sprague Dawley (SD) rats (n=5) for 4 days before the induction of ischemic stroke

using endothelin 1 (ET-1). It was found the consumption of BEF pre-ischemic stroke induction could significantly confer protection to the brain tissue against the ischemic injury, shown by the significantly improved neurological deficits, lower brain infarct volume and lower histological score of neuronal degradation in BEF-treated group, compared to the non-treated group. In addition, this study also evaluated the potential of BEF as preconditioning agent to confer protection to neural stem cells (NSCs) against ischemic conditions before transplantation into ischemic stroke rat models for treatment. The results revealed that the experimental rats treated with NSCs preconditioned with BEF at 3.125 μg/mL for 48 hours not only showed significantly decreased brain infarct volume, neuronal degradation and inflammatory cells infiltration, they also showed significantly increased blood vessel density and improved neurological behavioral function as fast as just 24 hours after the treatment, compared to rats treated with non-preconditioned NSCs and non-treated control group. Furthermore, the expression for angiogenic (ANGPT1), anti-oxidant (SOD2), anti-inflammation (IL-1Rn) and neuroprotective (JAKMIP1, STAT6, NGF, NFKβ) genes also significantly increased in the rat treated with BEF-preconditioned NSCs. In conclusion, BEF is a potential drug with neuroprotective and preconditioning effects that could be applied to enhance clinical treatments of ischemic stroke in future.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Stroke, a serious life-threatening global health problem, constitutes a major cause of mortality and severe adult disability worldwide. Globally, stroke was ranked as a second-leading cause of death after ischemic heart disease, with an annual mortality rate up to 6.5 million (*Global Stroke Fact Sheet 2022*, 2022). There are two major types of stroke, namely ischemic stroke and hemorrhagic stroke. Globally, the ischemic stroke is more common as compared to the hemorrhagic stroke, in which it account for 80% of all reported stroke cases (Donkor, 2018). The onset of ischemic stroke involves a series of interrelated and coordinated ischemic events starting from occlusion of cerebral blood vessel, which lead to the loss of oxygen and nutrient supply, followed by energy failure, excitotoxicity, oxidative stress, disruption of blood-brain barrier (BBB), inflammation by pro-inflammatory cytokine and ultimately irreversible ischemic damages at the affected brain region (Kuriakose and Xiao, 2020). Up to date, there is only one pharmacological drug, known as recombinant tissue plasminogen activator (rt-PA or alteplase), was approved by the United State Food and Drug Administration (FDA) for treatment of ischemic stroke (Imran, Mohamed and Nahab, 2021). Numerous clinical studies had proved that intravenous administration of rt-PA within 3 to 4.5 hours from the initial onset of acute ischemic stroke symptoms could significantly improve the clinical outcomes among the patients (Reed, Kerndt and Nicolas, 2022). Nonetheless, ironically, only about one-third of the ischemic stroke patients manage to arrive at the hospital for treatment within 4.5 hours after the symptom onset (Pan and Shi, 2021). As a consequence, majority of the patients are

deprived from the benefits of rtPA therapy and suffering from long term and severe ischemic stroke clinical outcomes (Mei *et al.*, 2019). Therefore, it is essential that every efforts are employed in finding novel therapeutic compound against the ischemic stroke disease.

In recent years, there is enormous emphasis on the development of novel therapeutics agents with high efficacy and low side effects from natural resources. *O. indicum*, locally known as 'Beko', is one of the medicinal plants in Malaysia that has been traditionally formulated to treat numerous ailments. Documented scientific findings of this plant revealed promising potential in its neuroprotective, anti-inflammatory, anti-oxidant, anti-diabetic, anti-cancer and many more beneficial biological properties that may contribute to the development of natural-based drugs from *O. indicum* (Nik Salleh *et al.*, 2020). The chemical constituents screening from different parts of *O. indicum* revealed that baicalein, a type of flavonoid compound as the most dominant compound of *O. indicum* (Dinda, Silsarma, *et al.*, 2015). Baicalein was also found to possess neurogenesis and neuroprotective properties (Nik Salleh *et al.*, 2020). However, before this compound can be used as a therapeutics agent in humans, it is essential to evaluate its safety profiles.

Safety profiles of natural compound are critical in the development of natural-product based therapy, as the prolonged unmonitored consumption of medicinal herbs might cause undesired effects to the general well-being. Therefore, regulatory authorities, under Kefauver-Harris Amendments 1962, an amendments from the previous Food, Drug and Cosmetic Act of 1938, has mandated that any new drugs or

substances must undergo rigorous toxicity testing on animals to ensure its safety (Kinch, Kinch and Griesenauer, 2019). The toxicity testing, starting with the determination of lethal dosage 50 (LD_{50}) in acute study, to the various long-term repeated dose toxicity testing, will provide detailed possible risk assessment of the tested substances (Ernest, Chibueze and Emmanuel, 2018). As baicalein possesses the ability to cross BBB (Fong, Wong and Zuo, 2014), it is very important to test the safety profiles of baicalein before utilizing it as a novel therapeutics compound for ischemic stroke treatment. Thus, in this study, baicalein-enriched fraction (BEF) extracted from the *O.indicum* was tested in acute toxicity and subacute neurotoxicity study to evaluate its possible side effect on the central nervous system (CNS). Following this, the neuroprotective effects of BEF were tested orally as a therapeutic agents in ischemic stroke rats to aid the recovery of neurological function deficits caused by the ischemic stroke onset.

Aside from its efficiency as therapeutics agents, where the BEF was directly fed to the ET-1 ischemic stroke rats, this study also investigated the effects of the BEF as preconditioning agent to enhance the stem cells-based therapy for ischemic stroke treatment. Stem cell-based therapy has shifted as a potential therapy for adult tissue regeneration and replacement of diseased, dysfunctional or injured tissue (Aly, 2020). Stem cells and their derivatives can be manipulated to specialize into specific types of cells, where it exhibits a promising clinical application compared to conventional whole organ transplantation which usually has higher risk and limited supply. Treatment on ischemic stroke utilizing stem-cell based therapy has used several types of cells, including neural stem cell (NSCs) (Chrostek *et al.*, 2019). In fact, various preliminary studies and undergoing NSCs-based clinical applications for

neurodegenerative disease have been conducted, showing it's feasibility and safety (Gioia *et al.*, 2020). However, translations of stem cell-based therapy to transplantation clinical application are not easy, as these therapies possess limitations. Hostile microenvironment influenced by the inflammatory response mediators, coupled with the production of reactive oxygen species (ROS) post-ischemic stroke, have hampered the effectiveness of stem-cell based transplantation strategy (Berlet *et al.*, 2021). Thus, several remedial approaches to bridge the gap between the bench and the bedside that includes preconditioning of NSCs using hypoxic environment, electrical stimulation and natural compound, have been suggested for better transplantation outcomes (Othman and Tan, 2020). However, up to date, no *in vivo* study involving preconditioning of NSCs using baicalein for ischemic stroke treatment was reported. Therefore, this study elucidated the preconditioning of neural stem cells using baicalein to enhance the therapeutic potential of NSCs for ischemic stroke. The precise mechanism by which baicalein and NSCs remodel the brain in ischemic stroke rat model was also investigated using real time polymerase chain reaction (RT-qPCR) to determine the expression of genes related to ischemic stroke recovery.

1.2 Problem statement

The effectiveness of baicalein, a neuroprotective compound extracted from *O. indicum* was investigated as an alternative therapeutics agent for ischemic stroke treatment. However, lacking of safety profiles on this compound requires extensive toxicological evaluation before it can be used as a novel therapeutics agent in the ischemic stroke treatment. Thus, acute toxicity and subacute neurotoxicity study has been conducted in this study to assess any potential risk of baicalein on human health. Subsequently, the roles of baicalein as therapeutic agent was also investigated, where

its effects on neurological function, infarct volume and histopathology were evaluated. This study also expand the roles of baicalein as preconditioning agent in NSCs, to address the major hurdle of regenerative therapy, which is the extremely low survival rate of the NSCs after transplantation into the hostile microenvironment in an ischemic brain. Preconditioned NSCs using baicalein not only elucidate its effects on neurological function, infarct volume and hispathology, its underlying mechanism was also investigated to provide detailed validation for future application of baicalein in ischemic stroke therapy.

1.3 Significance of study

O. indicum plant is native in Malaysia, thus the source of baicalein is abundance in Malaysia, making it highly promising candidate for the development of an alternative treatment for ischemic stroke disorder. This study was designed to provide detailed insight of the potential toxicological effects and safety profiles of baicalein, as well as its neuroprotective effects in ischemic stroke rats when administered orally. This study also elucidates the potential of baicalein enriched from *O.indicum* to enhance the therapeutic potential of NSCs for ischemic stroke therapy. The baicalein-preconditioned NSCs were transplanted into ischemic stroke rat model and the improvement in the neurological deficit and specific histopathological score in animal model were compared to non-preconditioned NSCs.

1.4 Research objectives

1.4.1 Main objective

The main objective of the study was to investigate the role and mechanism of baicalein as therapeutics and preconditioning agents for ischemic stroke rat model recovery *in vivo*.

1.4.2 Specific objectives

The specific objectives of the study includes:

1. To extract, fractionate and characterize baicalein-enriched fraction (BEF) from *O. indicum* plant.
2. To determine the safety profile of direct oral feeding of BEF in rat models using *in vivo* acute and subacute neurotoxicity tests.
3. To treat ischemic stroke rat models using direct oral administration of BEF and assessment of the ischemic stroke recovery after the treatment.
4. To determine the cytotoxicity effects and optimum concentration of BEF for NSC preconditioning *in vitro*.
5. To investigate the effects of BEF-preconditioned NSC transplantation on neurological functions and brain tissue morphology remodelling of ET-1 ischemic stroke rat models.
6. To determine the key genes in regulating ischemic stroke recovery after the BEF-preconditioned NSC transplantation.

CHAPTER 2

LITERATURE REVIEW

2.1 Stroke

Stroke, a serious neurological disease, is the second commonest cause of death and long-term adult disability globally after ischemic heart disease. Annually, about 15 million people worldwide are affected, with more than 6.5 million people died and another 5 million people permanently disabled due to stroke (World Health Organization (WHO), 2022). In Malaysia, stroke is presently among the top three leading cause of death (8.3%) and hospitalization after ischemic heart disease (17%) and pneumonia (11.4%), out of the 43, 237 medically certified death in 2020, particularly among the middle age and elderly group (Department of Statistic Malaysia, 2022). The prevalence of stroke is expected to rise significantly across the globe, largely due to the increasing number of older population (> 65 years old) by approximately 9 million people per year (United Nations, 2019).

Based on the American Stroke Association, stroke is defined as an 'acute neurologic dysfunction due to the disturbance in the cerebral blood supply, with acute onset of clinical signs and symptoms within minutes and persisted for more than 24 hours or leading to death' (American Stroke Association, 2013). Generally, there are two main classification of stroke, namely ischemic or hemorrhagic stroke (Unnithan, Das and Mehta, 2023). Ischemic stroke is triggered by focal cerebral ischemia, most frequently caused by the occlusion of cerebral artery (DeSai and Shapshak, 2022) or stenosis (Shukla *et al.*, 2017). Ischemic stroke can cause neuronal cell death due to the deprivation of oxygen and nutrient supply to the cells. On the other hand, hemorrhagic

stroke occurs mainly due to the spontaneous rupture of blood vessels or cerebral aneurysm in the brain which causes blood spilling into the spaces surrounding the brain cells (Unnithan, Das and Mehta, 2023). Hemorrhagic stroke can cause physical damage to the brain due to the buildup of pressure within skull. Among these two main type of stroke, ischemic stroke is the most common stroke which makes up about 85% of total reported stroke cases (Murphy, 2020).

2.1.1 Ischemic stroke

Ischemic stroke occurs when cerebral blood flow (CBF) of the CNS is suddenly reduced or interrupted by occlusion, causing focal neurological deficit (Kuriakose and Xiao, 2020). CBF that are less than 10-12 mL/100 g of brain tissue per minute at the core of infarction will lead to irreversible neuronal injury within an hour, whilst a complete obstruction of CBF will cause permanent death of brain tissue within just 4-10 minutes. While stroke incidence is influenced by sex, with men generally experiencing a higher incidence throughout their lives, women have a higher overall stroke prevalence due to an increased risk of stroke with age and longer lifespan compared to men (Roy-O'Reilly and McCullough, 2018). Neurological sign and symptom of an ischemic stroke usually appear suddenly, but less frequently, they can also occur in a progressive manner (Chong, 2022). Clinical symptoms and extent of ischemic injury varies depending on the rate of onset and duration, state of collateral circulation, health of systemic circulation, hematological factors, body temperature and glucose metabolism (Maida *et al.*, 2022). Common etiology that results in cerebral ischemia stroke include thrombosis or artherosclerosis (50%), microartery occlusion (25%) and embolism (25%) (Thaler and Fara, 2023).

Thrombotic ischemic stroke (Figure 2.1A) is more common in the elderly, and could occur without warning in more than 80% of cases (Yousufuddin and Young, 2019). However, there are also certain cases of transient ischemic attack (TIA) reported a few months before the actual ischemic stroke attack (Lioutas *et al.*, 2021). TIA is a temporary period of symptoms similar to those of a stroke that usually lasts only a few minutes and doesn't cause permanent damage. A TIA could act as a warning of a future stroke because almost one-third of patients who has had a TIA will eventually have a stroke (Supreet, 2016). The pathophysiology of thrombotic ischemic stroke is caused by formation of thrombus (blood clot) in larger cerebral artery, such as internal carotid artery or proximal and intracranial vertebral arteries from an aggregation of platelets and coagulation of fibrin at the vascular wall (atherosclerosis) (Bacigaluppi *et al.*, 2019). This thrombus could occlude the artery, forming an infarct (lacune), typically at basal ganglia, thalamus, pons, cerebellum or internal capsule of brain (Aggarawal *et al.*, 2010).

Another types of ischemic stroke is known as lacunar stroke (Figure 2.1B). It is smaller infarct located deeper in the cerebrum and brainstem of the brain, usually due to the occlusion of small penetrating branches (measured less than 20 mm in diameter) of large artery. This type of ischemic stroke is almost entirely related to chronic hypertension (68%) and diabetes (30%) which causes abnormalities of small arterioles, making it more susceptible to occlusion (Yaghi *et al.*, 2021).

On the other hand, embolic ischemic stroke (Figure 2.1C) refers to the formation of embolus (clot) at another location in the blood circulatory system, usually originated from the cardiac or large arteries of the upper chest and neck. Embolic

stroke occurs when the clot breaks, loose and travels in the bloodstream, where it could wedge and block a medium-sized branching arteries in or leading to the brain (Diener *et al.*, 2022). Approximately, two-third of emboli are of atherosclerotic origin, and may partially or temporally obstruct blood flow to cerebral arteries causing TIAs. Commonly recognized cardiac sources for embolism include atrial fibrillation, sinoatrial disorder, recent acute myocardial infarction (AMI), subacute bacterial endocarditis, cardiac tumors, and valvular heart disease (Muhamed *et al.*, 2016). Besides clot, fibrin and pieces of atheromatous plaque, materials known to embolize into the central circulation such as fat, air, tumor or metastasis, bacterial clumps, and foreign bodies are also contributing to the formation of embolic ischemic stroke (Caplan and Amarenco, 2016).

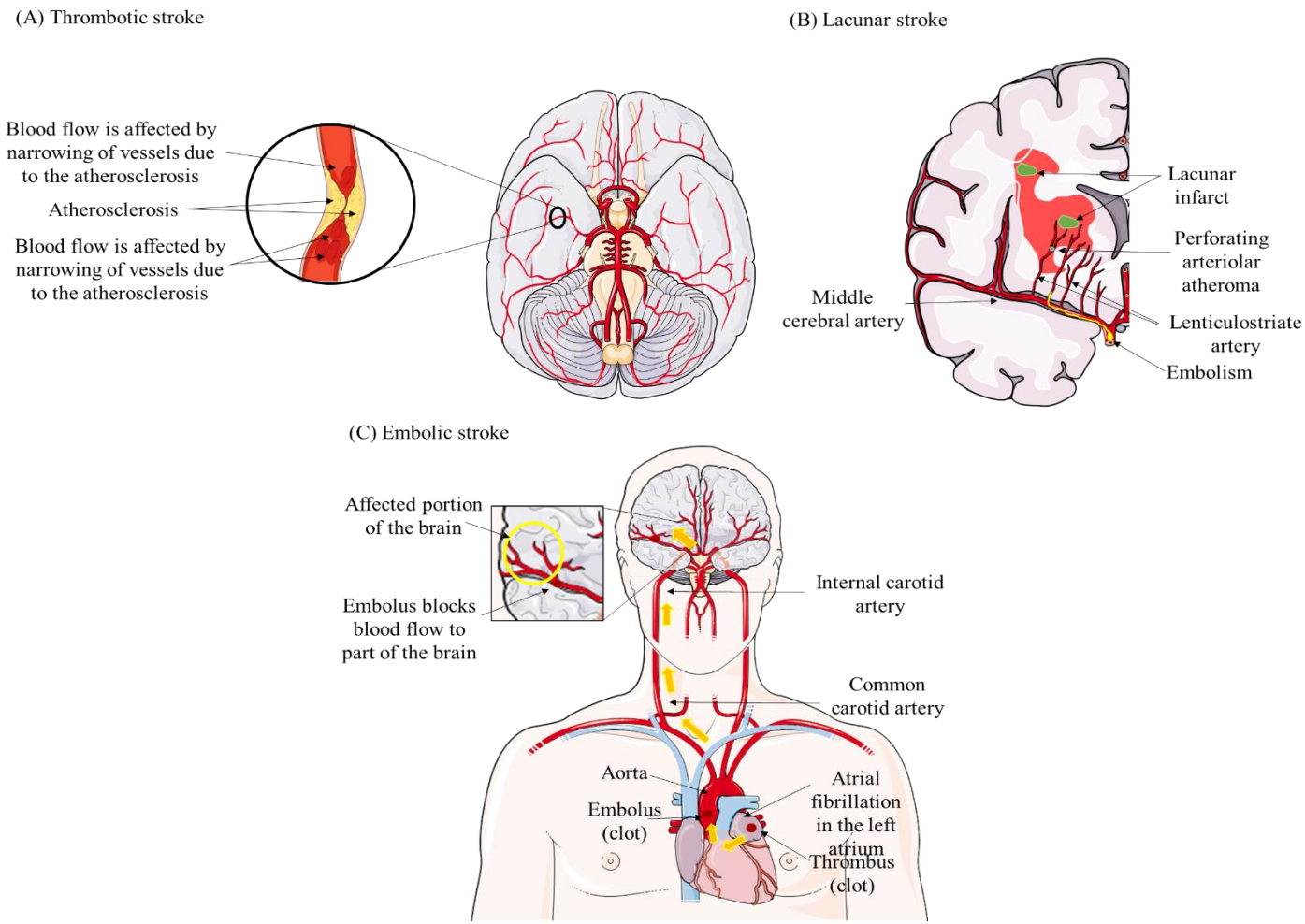

Figure 2.1 Types of ischemic stroke (A) Thrombotic stroke occurs when thrombus formed from atherosclerosis in cerebral artery (B) Lacunar stroke occurs when a clot obstructs blood flow in one of the small arteries located deep in the brain. (C) Embolic stroke occurs when clot dislodges from distant site of a brain, carried to and wedge in cerebral artery (modified from Mohd Satar, Othman and Tan, 2022).

2.2 Pathophysiology of neuronal injury in ischemic stroke

The development of hypoxic ischemic neuronal injury in all subtypes of ischemic stroke is greatly influenced by a series of interrelated and coordinated biochemical events that eventually lead to disintegration of cell membrane and neuronal death at the core of infarction. These biochemical events started with occlusion of blood vessel, followed by reduced brain nutrient, energy and oxygen, ionic imbalance, glutamate excitotoxicity, increase of intracellular calcium, oxidative and nitrosative stress which leads ultimately to neuronal cell death (Kuriakose and Xiao, 2020).

2.2.1 Reduced brain nutrient, energy and oxygen

Ischemic injury cascade is initiated by the occlusion of blood vessel which causes local depletion of oxygen and glucose supply, where it could progress to failure of energy production, particularly adenosine triphosphate (ATP) (Balcha *et al.*, 2020). The production of ATP occur in two conditions, either in the presence of oxygen (aerobic respiration) or in the absence of oxygen (anaerobic respiration). Under normal physiological condition (Figure 2.2A), aerobic respiration is the preferred method of glucose oxidation, namely cellular respiration, as this process produces more ATP (~92%) compared to the anaerobic respiration (Chaudhry and Varacallo, 2023). Aerobic respiration consisted of four processes, starting from glycolysis, pyruvate processing, tricarboxylic acid (TCA)/Krebs cycle and electron transport chain (ETC) coupled with mitochondrial oxidative phosphorylation process which require distinctive glucose and oxygen molecules to complete the processes (McLaughlin, 2020).

Glucose is first converted into pyruvate in glycolysis, producing 2 ATP and nicotinamide adenine dinucleotide (NAD) + hydrogen (NADH). Pyruvate enters the mitochondria and is processed into acetyl coenzyme A (acetyl-CoA), releasing carbon dioxide (CO_2) and generating more NADH. Acetyl CoA is further oxidized in the TCA cycle to produce more ATP, NADH, and flavin adenine dinucleotide (FADH2). The high-energy electrons from NADH and FADH2 are then transported through a series of protein complexes in the electron transport chain (ETC), located in the inner membrane of mitochondria. The energy released in this process is used to establish a proton gradient that powers ATP synthesis through ATP synthase (Manoj *et al.*, 2018). Following glucose and oxygen deprivation (OGD), glucose oxidation will switch from aerobic respiration to anaerobic respiration until no more glucose available (Figure 2.2B). Through this type of respiration, the number of ATP produced is far lower (only 2 ATP per glucose molecule) compared to the aerobic respiration (36 ATP per glucose molecule), making it to be less effective for energy synthesis in high energy demand brain (Sifat *et al.*, 2022).

Anaerobic respiration also cause a harmful side effects, as it cause intracellular acidification from the accumulation of its byproducts protons (H^+) and lactate. Intracellular acidification can eventually cause further decline in ATP concentration (Shegay *et al.*, 2022). As brain has high energy demand (at least 20% of total body's energy consumption), abundance of ATP are required to sustain its basal activities. During ischemic stroke, deprivation of oxygen and glucose directly impairs energy dependent process in severely affected ischemic regions, leading to domino-like cascade events triggering the cell death (Qin *et al.*, 2022).

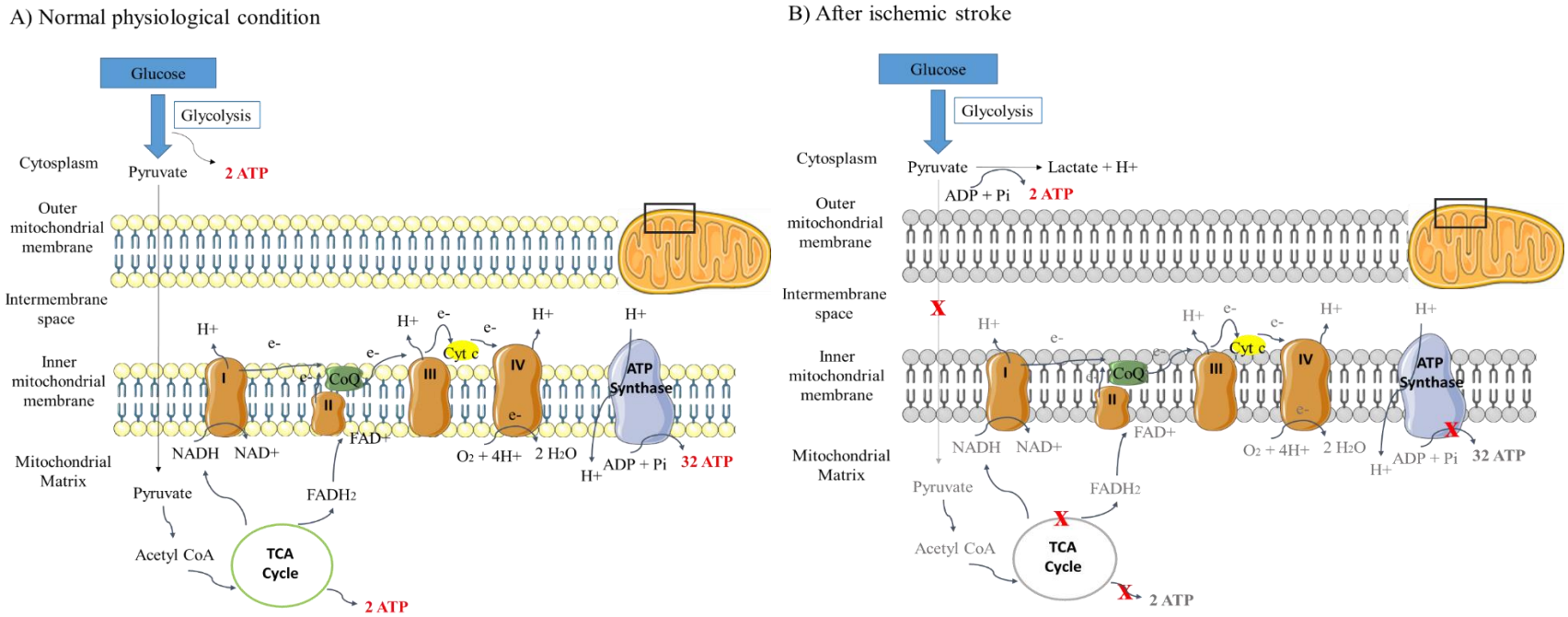

Figure 2.2 Cellular respiration during (A) normal physiological condition and (B) ischemic stroke condition. (A) In the presence of oxygen, glucose is oxidized through 4 important interrelated process, producing ATP via chemiosmosis process. (B) Under the deprivation of oxygen during ischemic stroke, glucose is processed via anaerobic respiration, due to the malfunction of pyruvate conversion process, TCA cycle and chemiosmosis, generating less ATP and causing harmful side effects of acidosis. ATP: Adenosine triphosphate; ADP: Adenosine diphosphate; TCA: Tricarboxylic acid; NADH: Nicotinamide adenine dinucleotide (NAD) + hydrogen; NAD^+: Nicotinamide adenine dinucleotide; $FADH_2$: Hydroquinone form of flavin adenine dinucleotide (FAD^+); FAD^+: Flavin adenine dinucleotide;O_2: Oxygen ; H_2O: Water ; H^+: Proton; e-: Electron; Pi: Phosphate, Cyt C: Cytochrome C.

2.2.2 Ionic imbalance

It has been observed that depletion of energy does not cause immediate cell death, but rather takes about 4 to 10 minutes to trigger a cascade of mechanism that lead to irreversible brain injury. One of the initial effects of energy failure after an ischemic onset is the malfunction of sodium-potassium (Na^+/K^+) pump gate which requires approximately 70% of the energy demand in the brain (Kirdajova *et al.*, 2020). Na^+/K^+ pump plays critical role to maintain the ionic gradient across the brain cell membrane for neuronal membrane potential (Pivovarov, Calahorro and Walker, 2018). Therefore, failure of the Na^+/K^+ pump results in ion imbalance, followed by water inflow, causing rapid cellular swelling of neurons and glia (cytotoxic edema) (Hellas and Andrew, 2021). Failure of ionic pumps also causing K^+ to flow out of the cell into the extracellular space. The altered K^+ gradient causes neuronal membrane depolarization, which opens voltage gated Na^{2+} and calcium (Ca^{2+}) channels and increases intracellular Na^{2+} and Ca^{2+} levels (Shattock *et al.*, 2015). Furthermore, failure of the ionic pump also can cause depolarization of neurons and astrocytes, which leads to excess release of neurotransmitter (particularly glutamate) that causes neuronal excitotoxicity.

2.2.3 Glutamate excitotoxicity

Excitotoxicity occurs due to the excess release of neurotransmitters, primarily glutamate along with excessive activation of their receptor (Armada-Moreira *et al.*, 2020). The ‘overreaction’ of this neurotransmitter is triggered by the ionic imbalance as explained in the previous section. Under normal physiological condition, glutamate is stored inside the synaptic terminal, where it will be cleared from the extracellular space by energy dependent process of N-methy1-D-asapartate receptor (NMDAR),

particularly GluN2A subunit which is dominantly located at synaptic sites (Figure 2.3A) (Mahmoud *et al.*, 2019). It has been suggested at GluN2A NMDAR subunit activate the pathways responsible for cell survival signaling. However, during acute ischemia, GluN2A NMDAR dysfunction to revert glutamate back to the inactive state, causing excitatory neurotransmitter glutamate accumulation at the synaptic cleft and extracellular space, where it will further cause neuronal membrane depolarization, which opens voltage gated Na^{2+} and Ca^{2+} channels and increases intracellular Na^{2+} and Ca^{2+} levels (Shattock *et al.*, 2015) (Armada-Moreira *et al.*, 2020). Overstimulation and persistent membrane depolarization by glutamate causes further influx of Na^{2+} and Ca^{2+} ions, and efflux of K^{+}, further deteriorate the ionic imbalance and glutamate excitotoxicity. This condition will leads to activation of cell death signaling via GluN2B at extrasynaptic sites, causing neuronal cell death (Figure 2.3B) (Girling and Wang, 2016).

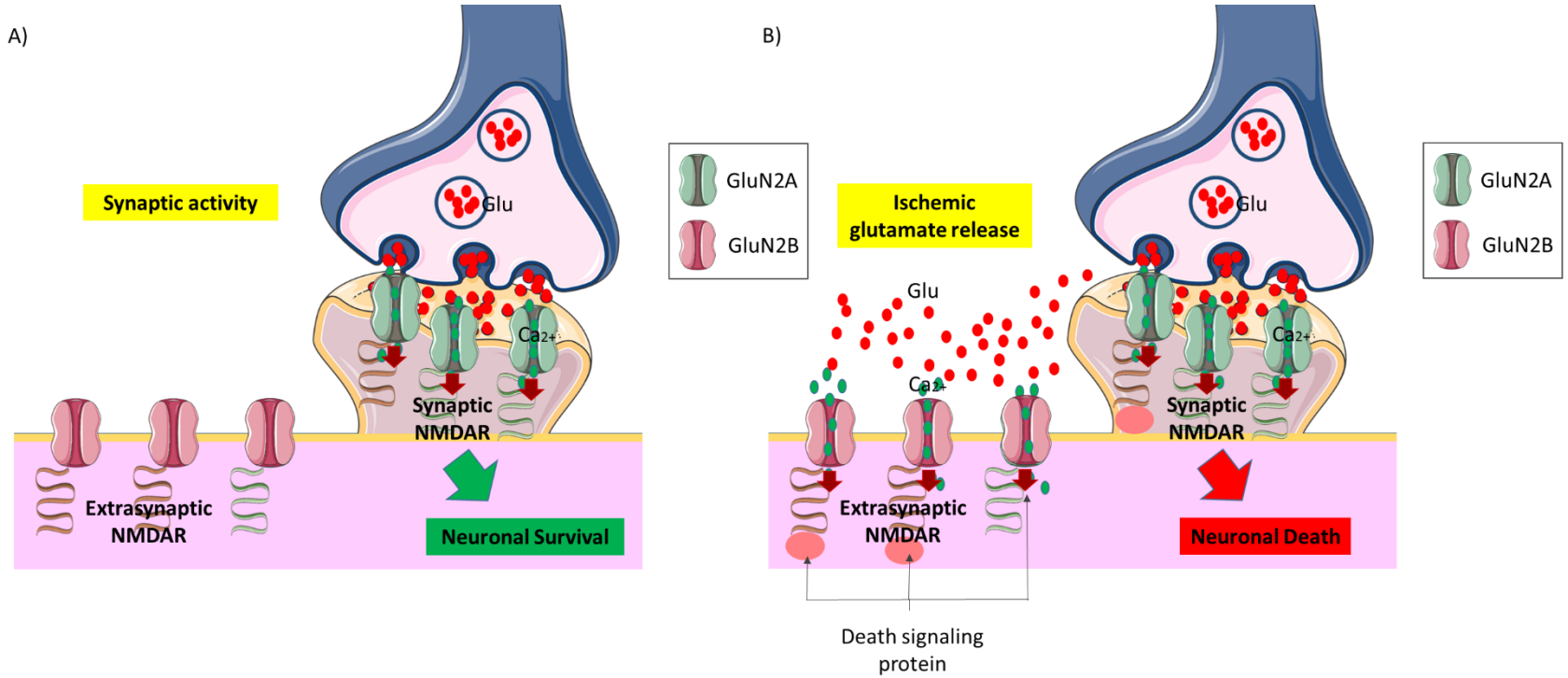

Figure 2.3 Glutamate expression at synaptic terminal under (A) normal condition and (B) ischemic stroke condition. (A) Under normal condition, glutamate is cleared from extracellular space via activation of the GluN2A NMDAR subunit. This pro-survival effect is dependent on the calcium influx through the receptors. (B) During cerebral ischemia, failure of ionic pumps cause NMDAR dysfunction to revert back to inactive state, causing excessive accumulation of glutamate at synaptic cleft and extracellular space which further deteriorates the ionic imbalance and glutamate excitotoxicity, leading to activation of GluN2B subunit that recruit death signaling protein, inducing neuronal cell death (Figure modified from Girling and Wang, 2016).

2.2.4 Increase in intracellular calcium

Following the glutamate excitotoxicity, the influx of Ca^{2+} into cytoplasm activates the destructive enzymes such as protein cam-kinase II and protein kinase C that stimulate protein phosphorylation, resulting in destabilization of neuronal homeostasis (Yaghi *et al.*, 2021). Other series of damaging enzymatic pathway activated by Ca^{2+} including calpain, a protease which causes cytoskeletal proteolysis and phospholipase A, a lipase which leads to production of arachidonic acid and free radical formation. The consequences of free radical production and these enzyme perturbations are widespread including disruption of neuronal (and endothelial) membrane and cytoskeletal integrity as well as damage to mitochondrial function (Ludhiadch *et al.*, 2022).

2.2.5 Oxidative and nitrosative stress

Recent studies suggest that ischemic injury are closely linked to induction of oxidative stress, a condition related to an increased rate of cellular damage induced by oxygen-derived free radicals, or commonly known as reactive oxygen species (ROS) (Ludhiadch *et al.*, 2022). Although Ca^{2+} does not have a direct impact on the function of the respiratory chain or the oxidation/reduction processes, excessive accumulation of Ca^{2+} in the mitochondria can results in an elevation of ROS (Peng and Jou, 2010). ROS is highly reactive oxidizing agents, with one or more unpaired electrons belongs to the group of free radicals. It is produced when there is an imbalance between the production of free radicals and scavenging capacity by antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione (Vona *et al.*, 2021). Compared to other tissues and organs, brain is particularly sensitive to oxidative stress-induced damage because of its high concentration of unsaturated lipids and iron that act as pro-

oxidant during stress. Besides, its high consumption of oxygen under basal condition and its relatively low endogenous antioxidant capacity also makes it a vulnerable organ to oxidative stress (Lee, Cha and Lee, 2020). Following ischemic stroke injury, superoxide (O^{2-}) anion, a primary species of ROS is produced by mitochondria during electron transport process and also by metabolism of arachidonic acid through the lipooxygenase and cyclooxygenase pathways (Su *et al.*, 2019).

Excessive nitric oxide (NO) gases molecule is synthesized from L-arginine through activation of several NO synthase (NOS) isoforms. The neuronal isoform (nNOS) is expressed in neuronal cell bodies, where NO derived from nNOS are required for memory formation, release of neurotransmitter, transmission of pain signals and for regulation of central nervous system blood flow (Iova *et al.*, 2023). Activation of NMDAR by glutamate also stimulate intracellular NO production by nNOS, and to possibly induce excitotoxic-mediated injury in the ATP depleted post synaptic cell (Chamorro *et al.*, 2016). The second NOS isoform is called inducible NOS (iNOS). It is expressed by inflammatory cells such as microglia and monocytes in response to inflammatory cytokines or endotoxin, where it has larger amount of NO produced compared to nNOS and endothelial cell isoforms (eNOS), due to its independence from calcium dependant mechanism for activation (Xue *et al.*, 2018). These two isoforms are, for the most part, damaging the brain under ischemic conditions. The third isoform is endothelial NOS (eNOS), where NO produced by eNOS has vasodilatory effects and is likely to play a beneficial role in maintaining the brain microcirculation, reducing smooth muscle proliferation, inhibit platelet aggregation and for leukocyte adhesion and migration (Tran *et al.*, 2022). All NO produced will diffuse freely across membranes and react with O^{2-} anion at its point of

generation to produce peroxynitrite ($ONOO^-$), another highly reactive oxygen species that cause protein nitration and dysfunction. Both oxygen-derived free radicals and reactive nitrogen species are involved in activating several pathways involved in promoting cell death directly or indirectly following stroke, such as apoptosis and inflammation (Chavda *et al.*, 2022).

2.2.6 Neuronal death

Within a few minutes after cerebral artery occlusion, the core of brain tissue exposed to the reduced blood flow will undergo mortally injured and necrotic cell death. Necrosis in the core region is morphologically characterized by initial cellular and organelle swelling, subsequent disruption of plasma membranes, nuclear chromatin condensation (pyknosis) and ultimately release of cell contents into the extracellular space (Hu *et al.*, 2021).

In contrast to necrosis, apoptosis (programmed cell death) occur only after several hours or days after the onset of stroke and may have a potential of recovery after blood flow restored. Apoptosis causes nuclear damage, whilst maintaining the integrity of plasma and mitochondrial membrane until late in the process, where it allowed the cells to die without eliciting inflammatory response to neighboring cells (Galluzzi *et al.*, 2018). Activation of specific gene leading to cell death by apoptosis, such as caspases can occur via intrinsic or extrinsic pathway (Jan and Chaudhry, 2019). Intrinsic or death receptor-independent pathway are initiated by the release of cytochrome C from mitochondria, resulting in activation of caspase-9 and subsequently caspase-3. Meanwhile, extrinsic or death receptor-dependent pathway are triggered by activation of cell surface death receptors, also from mitochondria

resulting in activation of caspase-8 (Tang *et al.*, 2019). Both pathways cause autolytic process mediated by deoxyribonucleic acid (DNA) cleavage, resulting in the destruction of cell (Miller and Zachary, 2017).

2.3 Signaling pathways involved in ischemic stroke

In recent years, significant research have been taken to unravel the underlying pathophysiological mechanisms of ischemic stroke, which involves multitude of signaling pathways. These pathways, some of which can be detrimental, while others may confer neuroprotection, intricately intersect and give rise to a complex signaling network within the forementioned pathophysiology. As some of these signaling pathways offer promising therapeutic avenues, targeting them holds the potential to serve as an effective strategy against ischemic stroke. Among the patways extensively investigated in ischemic stroke are phosphatidylinositol 3-kinase (PI3K)-Akt, phosphatase and tensin homolog (PTEN), death-associated protein kinase 1 (DAPK1), hypoxia-inducible factor (HIF), nuclear factor E2-related factor 2 (Nrf2), casein kinase 2 (CK2) beclin1/Bcl2 and Notch (Qin, Yang and Chu, 2022). Each of these pathways plays a crucial role in intervening the cascade of events leading to neuronal cell death. In this study, JAKMIP1-STAT 6-GABRA6 pathway, known for its involvement in the regulation of voluntary movements (Lee, Ueno and Yamashita, 2011), was investigated. Additionally, we also explored the NGF-NF-κβ pathway, which plays a critical role in control of cells growth, proliferation and survival (Shih, Wang and Yang, 2015).

2.4 Current ischemic stroke treatment and its limitation

As most strokes are largely caused by thromboembolic occlusion, restoration or improvement of perfusion to the ischemic lesion area has become the key therapeutic strategy to treat this disease. This is because there are possibility of restoring area surrounding the core of infarction if the blood flow is restored as soon as possible. This area, known as ischemic penumbra region, is only affected at a level within the functional impairment thresholds and still remain metabolically active where it may be functionally recovered if blood circulation is successfully restored within the therapeutic window (usually between 3 to 4.5 hour, depending on the severity of ischemia) (Ermine *et al.*, 2021).

At present, the only FDA-approved drug recommended for restoration of blood flow in ischemic stroke treatment is intravenous rtPA. This drug dissolve the blood clot by activation of plasminogen, where it will form plasmin (Izadi *et al.*, 2021). Plasmin is essential proteolytic enzyme that will break cross-links between fibrin and lessen the damage caused by blockage of blood vessel (Jilani and Siddiqui, 2023). However, this thrombolytic drug is associated with increased risk of fatal intracranial hemorrhage (6.3%), narrow therapeutic window (<4.5h), high re-incidence rate and short half-life (<5 min), making the application of this drug to be limited (Mei *et al.*, 2019).

Aside from the thrombolytic drug, other choices of treatment for ischemic stroke includes anticoagulants and antiplatelet agents (Bir and Kelley, 2021). Anticogulant drugs such as heparin and warfarin prevent blood clotting by interfering with the coagulation cascade. However, these drugs do not dissolve the existing clot

or reversing ischemic tissue damage, and their use in the acute phase of ischemic stroke may increase the risk of bleeding complications (Crader, Johns and Arnold, 2023). Meanwhile, antiplatelet drugs such as aspirin and clopidogrel, prevent platelet aggregation and blood clot formation. While they can reduce the risk of stroke in some cases, they also have increased risk of bleeding and dyspepsia, and not as effective as rtPA in dissolving existing clot (Kapil, Datta and Alakbarova, 2017).

Another option for ischemic stroke treatment involves the usage of endovascular mechanical thrombectomy, which is a minimal surgical wise procedure to insert a catherer into the affected blood vessel in the brain to remove the blood clot. This procedure was performed under imaging guidance to guide the placement of the catherer. However, as this procedure require specialized equipment and trained personnel, with certain risk and complications such as bleeding, infection or damage to the blood vessel, it would limit the application of this procedure (National Guideline Centre (UK), 2019). Apart from the aforementioned treatments, there have been no breakthrough in stroke treatment for decades, until recently where some emerging promising researches has been done using safer, multi-targeted natural products approach combined with regenerative therapy for ischemic stroke treatment.

2.5 Natural product-based therapy

Natural products derived from medicinal plants have been proposed as an important biological sources for novel neuroprotective agent discovery. This is mainly because these plants contains various potential phyto-constituents that could target multiple mechanisms of actions in neuroprotection approaches. The multi-target natural product-based therapy could provide promising strategies for the prevention

and cure of multifactorial pathological mechanisms associated with ischemic stroke as described in earlier section (Tao *et al.*, 2020).

2.5.1 Medicinal potency of *Oroxylum indicum*

Since past decades, numerous studies have demonstrated the promising effects of crude extracts and isolated bioactive compounds on brain neuroprotection from different types of plant materials. In this study, one of the most potential medicinal plants, namely *O. indicum*, was chosen for further investigation.

O. indicum is an edible medium-sized tree originated from Biognoniaceae family. In Malaysia, it is known as 'Beko' by the local population. It is also known as "Midnight Horror" because the flowers produce unpleasant smell that functioned to attract the bats to facilitate the pollination process. This plant is native in Southeast and South Asian region areas including China, Indonesia, Malaysia, Philippines, Sri Lanka, Thailand and India (Devanathan, 2021), where it can be found in tropical and subtropical low-altitude forest.

O. indicum is a medium sized tree, typically reaching heights between 8 to 15 meters, which branches at the top (Figure 2.4). The bark of this tree is light brown, soft, and contains green juice, often exhibiting numerous corky lenticles. The leaves of the tree are pinnate, measuring approximately 3 to 7 cm in length, with opposite pinnae and a very stout, cylindrical rachis. The leaflet are typically 2 to 4 pairs, measuring 6 to 12 cm in length and 4 to 10 cm in width, with an ovate or elliptic shape and acuminate tip (Figure 2.4A) (Dinda, SilSarma, *et al.*, 2015). The tree produces a large number of foetid flowers that are reddish purple outside and pinkish yellow