TRANSCRIPTOME ANALYSIS OF ISCHEMIC STROKE RECOVERY INDUCED BY NEURAL STEM CELL PRECONDITIONED WITH BAICALEIN-ENRICHED FRACTION OF *OROXYLUM INDICUM*

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by

ASMAA' BINTI MOHD SATAR

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LIST OF EQUATIONS

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LIST OF SYMBOLS

<	Less than
>	More than
-	Minus
%	Percentage
α	Type I errors
β	Type II errors
°C	Degree Celsius
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
ip	Intraperitoneal
K^+	Potassium
kg	kilogram
Na^{2+}	Sodium
mg	milligram
mL	Milliliter
mm	Millimeter
mM	Micromolar
ng	Nanogram

nm	nanometer
O_2^-	Superoxide
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

ABCC9	Keratin associated protein 3-2
AGCC	Affymetrix GeneChip Common Console
ANOVA	Analysis of variance
AOAH	Acyloxyacyl hydrolase
APE 1	Apyrimidinic Endonuclease
ARASC	Animal Research and Service Centre
ASD	Autism Spectrum Disorder
AUC	Area under curve
BBB	blood-brain barrier
BCR	B-cell receptor
BEF	baicalein-enriched fraction
bFGF	basic Fibroblast Growth Factor
BOC	BOC cell adhesion associated, oncogene regulated
CALCB	calcitonin-related polypeptide, beta
CAMK2D	calcium/calmodulin-dependent protein kinase II delta
cAMP	Cyclic adenosine monophosphate
CAR8	Carbonic anhydrase 8
CARD11	caspase recruitment domain family, member 11
CBLN1	Cerebellin 1 precursor
CBLN3	Cerebellin 3 precursor
CDHR1	cadherin-related family member 1
cDNA	Complementary deoxyribonucleic acid
CGH	comparative genomic hybridization
CNS	central nervous system

CO2	Carbon dioxide
cREB	cAMP response element-binding protein
cRNA	Complementary ribonucleic acid
CST	Corticospinal tract
CVA	cerebrovascular disease
DALYs	disability-adjusted life years
DAT	Dopamine transporter
DAVID	Database for Annotation, Visualization and Integrated Discovery
DEG	differential expression genes
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco phosphate buffer saline
DPSCs	dental pulp stem cells
DRD2	dopamine receptor D2
DRD3	Dopamine receptor D3
ds-cDNA	Double stranded complementary deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ET-1	Endothelin-1
F5	Fraction 5
FAT2	FAT atypical cadherin 2
FCGR2B	Fc gamma receptor 2B
FCGR3A	Fc gamma receptor 3A
FDA	Food and Drug Administration
FGF1	fibroblast growth factor 1
FGFR3	fibroblast growth factor receptor 3

GABA	γ-Aminobutyric acid
GABRA6	Gamma-aminobutyric acid type A receptor subunit alpha 6
GLRA	glycine receptor, alpha 1
GNG3	G protein subunit gamma 3
GNG4	G protein subunit gamma 4
GO	Gene Ontology
GPR88	Keratin associated protein 3-3
GRID2	glutamate ionotropic receptor delta type subunit 2
HCl	hydrochloric acid
HIST2H3C2	Histone cluster 2 H3 family member C2
HPLC	High-performance liquid chromatography (
HRPT1	Hypoxanthine phosphoribosyltransferase 1
IGSF9B	Phosphodiesterase 10A
IL16	Interleukin 16
IL-1RN	Interleukin-1 receptor antagonist
IL1β	Interleukin-1-beta
IL33	Interleukin 33
INHBA	Immunoglobulin superfamily, member 9B
iPSC	Induced pluripotent stem cell
IVT	intravenous thrombolysis
IVT	*In vitro* transcription
JAKMIP1	Janus kinase and microtubule interacting protein 1
JAK-STAT	Janus kinases (JAKs), signal transducer and activator of transcription proteins (STAT)
KCNJ2	Inhibin subunit beta A
KEGG	Kyoto Encyclopedia of Genes and Genomes
KRTAP3-2	Olfactory receptor family 52 subfamily W member 1

KRTAP3-3	ATP binding cassette subfamily C member 9
LBP	enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase
LCN2	Lipocalin 2
LIFR	LIF receptor subunit alpha
LOC686660	Keratin associated protein 3-3
MAPK	Mitogen-activated protein kinase
MCA	middle cerebral artery
MEIS1	Meis homeobox 1
MGP	Matrix Gla protein
mNSS	modified neurological severity score
MPEG1	Macrophage expressed 1
MPZ	myelin protein zero
MS4A12	Membrane spanning 4-domains A12
MSCs	mesenchymal stem cells
MT	Mechanical thrombectomy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NDNF	Neuron-derived neurotrophic facto
NEUROD1	Neuronal differentiation 1
NF-β	Nuclear factor kappa B subunit 1
NGF	Nerve growth factor
NGFR	nerve growth factor receptor
NGFR	Nerve growth factor receptor
NSCs	neural stem cells
NSS-R	The Revised Neurobehavioral Severity Scale
OLR903	Olfactory receptor family 6 subfamily C member 35
PAX6	paired box 6

PBS	phosphate buffer saline
PCA	Principal component analysis
PCR	polymerase chain reaction
PDE10A	Potassium inwardly-rectifying channel, subfamily J, member 2
PIK3R3	phosphoinositide-3-kinase regulatory subunit 3
PKIB	cAMP-dependent protein kinase inhibitor beta
PMCH	Pro-melanin-concentrating hormone
QC	Quality check
qPCR	Quantitative real-time PCR
RET	ret proto-oncogene
RGS9	Inhibin subunit beta A
RIN	RNA integrity number
RNA	ribonucleic acid
ROS	reactive oxygen species
RPL13A	Ribosomal protein L13A
rt-PA	recombinant tissue plasminogen activator
SAPE	streptavidin phycoerythrin
SD	Sprague Dawley
SD	Standard deviation
SEM	Standard error mean
SFM	serum free medium
SGK1	Serum/glucocorticoid regulated kinase 1
SLC6A3	Solute carrier family 6 member 3
SNP	single nucleotide polymorphism
ss-cDNA	Single-stranded complementary deoxyribonucleic acid
STAT6	Signal transducer and activator of transcription 6
SVZ	subventricular zone

TAC	Transcriptome analysis console
TAE	Tris base-acetic acid-EDTA
TBE	Tris base-boric acid-EDTA
TdT	Terminal Deoxynucleotidyl Transferase
TH	tyrosine hydroxylase
TLR	Toll-like receptor
TNFRSF1A	TNF receptor superfamily member 1A
TNK	Tenecteplase
TRDN	Triadin
TTC	2,3,5-Triphenyltetrazolium chloride
UDG	Uracil-DNA glycosylase (
USM	Universiti Sains Malaysia
UV	Ultraviolet
VEGF	vascular endothelial growth factor A
WDR66	WD Receptor domain 66
WT	Whole transcriptome

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ANALISA TRANSKRIPTOM PEMULIHAN STROK ISKEMIA YANG TERARUH OLEH SEL STEM SARAF YANG DIPRAKONDISI DENGAN FRAKSI BAICALEIN *OROXYLUM INDICUM*

ABSTRAK

Strok iskemia merupakan salah satu punca utama kematian dan penyumbang utama kepada kecacatan di seluruh dunia. Transplantasi sel stem regeneratif yang telah diprakondisi dengan produk semulajadi telah digunakan untuk memulihkan saraf yang rosak selepas serangan strok iskemia. Walaubagaimanapun, pemacu kunci dan laluan yang mendasari pemulihan tersebut masih tidak diketahui. Dalam kajian ini, sel stem saraf (NSCs) yang telah diprakondisi dengan fraksi baicalein (BEF), sebatian aktif neuroprotektif yang diekstrak dari tumbuhan perubatan tempatan yang dikenali sebagai *Oroxylum indicum (O. indicum)*, telah ditransplankan ke dalam model tikus strok iskemia dan analisa transkriptom telah digunakan untuk mengekspresikan asid ribonukleik (RNA) otak untuk mengenal pasti gen dan jalan utama yang mendasari pemulihan stroke iskemia yang diinduksi oleh penanaman NSC yang telah diprakondisikan. Sejumlah 15 ekor tikus Sprague-Dawley (SD) disuntik dengan endothelin-1 (ET-1) untuk menghalang pembuluh darah arteri serebral tengah (MCA) di dalam otak, ianya menyerupai penyakit strok iskemia pada manusia. Model tikus strok iskemia yang diinduksi ET-1 ini dibahagikan secara rawak kepada 3 kumpulan, iaitu Kumpulan 1: Tidak dirawat (kumpulan kawalan, n = 5), Kumpulan 2: Dirawat dengan NSC yang tidak diprakondisi (n = 5), dan Kumpulan 3: Dirawat dengan NSC yang diprakondisi dengan BEF (n = 5). Tingkah laku neurologi haiwan dipantau dan dicatat berdasarkan ujian skor keparahan neurologi yang diubahsuai (mNSS), ujian silinder, dan ujian berjalan diatas grid selama 14 hari (p-value < 0.05). Selepas 14 hari,

semua tikus dimatikan melalui suntikan intraperitoneal ketamin (200 mg/kg) dan xilazin (20 mg/kg). Tisu otak dikumpulkan dan dibekukan dengan menggunakan nitrogen cecair untuk homogenkan tisu otak bagi tujuan pengekstrakan RNA. RNA yang diekstrak dianalisis menggunakan ujian mikroarray untuk mengenalpasti perbezaan gen (DEG), ontologi gen (GO), dan mekanisma biologi yang berkait dengan peningkatan tingkah laku neurologi tikus. Hasil kajian menunjukkan bahawa tikus yang dirawat dengan NSC yang prekondisi dengan BEF pada 3.125 µg/mL selama 48 jam meningkatkan fungsi tingkah laku neurologi secepat 24 jam selepas rawatan, (p-value < 0.05) berbanding dengan tikus yang dirawat dengan NSC yang tidak diprakondisikan dan kumpulan yang tidak dirawat. Selanjutnya, berdasarkan hasil mikroarray menunjukkan bahawa ekspresi GABRA6, NGF, JAKMIP1, DRD3, STAT6, NF-κβ, SLC6A3, dan IL-1RN telah diidentifikasi secara signifikan berdasarkan 10 teratas DEG, Gene Ontology (p-value < 0.05), dan laluan biologi menggunakan analisis laluan KEGG (p-value < 0.05). Laluan-laluan utama seperti laluan isyarat cAMP, laluan isyarat reseptor Toll-like, laluan isyarat reseptor B-cell, dan fagositosis termediasi oleh Fc gamma R berkaitan dengan peningkatan perilaku neurologi dalam model tikus strok iskemia. Secara ringkasnya, kajian ini menyediakan pengetahuan baru mengenai mekanisme rawatan NSC yang dipersiapkan dengan BEF untuk merawat strok iskemia berdasarkan ekspresi utama gen yang signifikan menggunakan analisis mikroarray.

TRANSCRIPTOME ANALYSIS OF ISCHEMIC STROKE RECOVERY INDUCED BY NEURAL STEM CELL PRECONDITIONED WITH BAICALEIN-ENRICHED FRACTION OF *OROXYLUM INDICUM*

ABSTRACT

Ischemic stroke is one of the leading causes of death and a major contributor to adult disability worldwide. Transplantation of regenerative stem cells preconditioned with natural products was applied to restore the damaged neural circuity after an attack of ischemic stroke. However, the key regulators and pathways underlying such recovery are still mainly unknown. In this study, neural stem cells (NSCs) preconditioned with baicalein enriched fraction (BEF), a neuroprotective active compound extracted from a local medicinal plant known as *Oroxylum indicum (O. indicum),* was transplanted into an ischemic stroke rat model and a transcriptome analysis was applied to profile the brain total ribonucleic acid (RNA) expression to identify the key genes and pathways underlying the ischemic stroke recovery induced by the preconditioned NSC transplantation. A total of 15 Sprague-Dawley (SD) rats were injected with endothelin-1 (ET-1) to occlude the middle cerebral artery (MCA) blood vessel inside the brain, mimicking the ischemic stroke disease in human. The ET-1 induced ischemic stroke rat models were randomly assigned into 3 subgroups, namely Group 1: Non-treated (control group, n = 5), Group 2: Treated with non-preconditioned NSCs (n = 5) and Group 3: Treated with BEF-preconditioned NSCs (n = 5). The animal neurological behaviours were monitored and scored based on modified neurological severity score (mNSS) test, cylinder test and grid-walking test for 14 days (p-value < 0.05). After 14 days, all the rats were sacrificed by intraperitoneal injection of ketamine (200 mg/kg) and Xylazine (20 mg/kg). The brain

tissues were harvested and snap-frozen using liquid nitrogen to homogenize the brain tissue for RNA extraction. The extracted RNA was analysed using microarray assay to reveal differentially expressed genes (DEGs), gene ontology (GO) and biological pathways related to neurological behavior improvement of the rats. The results revealed that the experimental rats treated with NSCs preconditioned with BEF at 3.125 µg/mL for 48 hours improved neurological behavioral function as fast as just 24 hours after the treatment (p-value < 0.05), compared to rats treated with non-preconditioned NSCs and non-treated group. Furthermore, based on microarray result showed the expression of GABRA6, NGF, JAKMIP1, DRD3, STAT6, NF-κβ, SLC6A3 and IL-1RN were significantly identified based on the top 10 of DEGs (p-value < 0.05), Gene Ontology (p-value < 0.05) and biological pathways using KEGG pathways analysis (p-value < 0.05). The key regulated pathways such as cAMP signaling pathway, Toll-like receptor signaling pathway, B-cell receptor signaling pathway and Fc gamma R-mediated phagocytosis were associated with the improvement of neurological behavior in the ischemic stroke rat model. In brief, this study provides new knowledge regarding the mechanism of BEF-preconditioned NSCs therapy to treat ischemic stroke based on the significant main expression of genes using microarray analysis.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Stroke is one of the global health burdens with increasing trend over the past few decades. Ischemic stroke, being the most common type of stroke, is causing almost 3.3 million deaths annually and it remains as the major causal factor of adult disability worldwide (Fraser *et al.,* 2023). Clinically, ischemic stroke is defined as a syndrome of acute, focal neurological deficits that occurs due to insufficient cerebral blood supply attributed to vascular injury of the central nervous system (CNS). The onset of ischemic stroke neuronal injury is initiated by the retardation of brain nutrients and oxygen, which triggers a series of interrelated and coordinated biochemical events that ultimately lead to neuronal cell death and neurological dysfunction (Hamblin *et al.*, 2021).

The tissue damage caused by ischemic stroke attack can be reduced if the blood flow occlusion is removed rapidly. Up-to-date, intravenous thrombolysis (IVT) with intravenous recombinant tissue plasminogen activator (rt-PA, alteplase) is the only approved pharmacological systemic therapy by the United State Food and Drug Administration (FDA) to remove blood clots in the ischemic stroke patients (Giulia & Maurizio, 2022). Even though this primary therapy could allow rapid restoration of blood supply and reduce stroke-related disability and mortality, this therapy is unable to regenerate new neuronal cells to replace the damaged brain tissue and thus brain function cannot be completely restored. The limitations of current ischemic stroke therapies have led to alternative therapeutic approaches such as using neural stem cells (NSCs)-based regenerative therapy to induce brain repair and remodeling in patients.

NSCs are multipotent cells which are able to generate progenitor cells that primarily differentiate into neurons, astrocytes, and oligodendrocytes to maintain brain homeostasis (Łos *et al.*, 2018). The NSCs possess a unique ability to mitigate stroke pathology since they can migrate through the CNS and repopulate lesion sites after ischemic injury (Jiang *et al.*, 2019). Therefore, NSC-based therapy has been recognized and highly expected as an effective strategy for treating degenerative brain diseases such as ischemic stroke (Hamblin *et al.*, 2021). Fundamentally, this therapy focuses on replacing dead cells in the infarcted area or enhancing the self-repair system by providing trophic support for reconstruction of neuronal cells. Nonetheless, when transplanted into the ischemic brain, these stem cells are forced to encounter with the hostile microenvironment at the acute ischemic site which is high in detrimental factors including reactive oxygen species (ROS) and inflammatory cytokines, resulting in a reduced stem cell engraftment rate, poor survival and lower proliferative abilities, which altogether limit the effectiveness of this cell-based therapy (Raziyeva *et al.*, 2020). In order to overcome the limitations of the cell-based therapy for ischemic stroke, preconditioned stem cells approach has been suggested to improve the stem cell survival rate and their therapeutic potential within the ischemic brain microenvironment prior to transplantation.

In this study, rat NSCs were preconditioned with baicalein-enriched fraction (BEF), a natural neuroprotective extract isolated from a local medicinal plant known as *Oroxylum indicum* or also known as Beko in Malaysia. Baicalein is a flavonoid compound that has been well known for its antioxidant, anti-inflammatory, anti-allergic, anti-virus, anti-bacteria, anti-cancer, as well as the neuroprotective properties (Nik Salleh *et al*., 2020). Here, the BEF-preconditioned NSCs were used to treat rat models induced with ischemic stroke using a vasoconstrictor known as endothelin-1

(ET-1). The ET-1 ischemic stroke rat model has been widely utilized because the occlusion of middle cerebral artery (MCA) and its branches by ET-1 is effective, reliable and capable of producing well-reproducible infarcts mimicking the human focal ischemic stroke (Hermann *et al*., 2019; Komatsu *et al.*, 2021). Neurobehavioral of the ET-1 ischemic stroke rat models were evaluated after the treatment with BEF-preconditioned NSCs and compared with those treated with non-preconditioned NSCs to determine the potential of BEF to enhance to the therapeutic potential of NSCs for ischemic stroke disease.

Additionally, a comprehensive transcriptomic analysis using microarray techniques was performed to profile total ribonucleic acid (RNA) expression in the animal brain tissue with and without BEF-preconditioned NSC treatments. This high-throughput transcriptome profiling enabled the identification of key regulator genes and pathways that mediated the neurological behavior changes as observed in the rat models. The elucidation key genes and pathways underlying ischemic stroke recovery induced by the BEF-preconditioned NSCs transplantation were valuable in assessing the potential of the BEF as an important natural compound supplement for stem cell-based ischemic stroke regenerative therapy in the future.

1.2 Problem statement

Ischemic stroke onset could trigger irreversible neuronal damage in patients afflicted with it. NSC-based therapy has been introduced as an alternative treatment for ischemic stroke because of its ability to self-renew and differentiate into matured functional neuronal cells. However, the treatment is limited by low cell survival after transplantation into the ischemic brain with hostile microenvironment high in ROS and inflammatory cytokines. A neuroprotective natural compound known as baicalein has

emerged as a potential candidate to precondition NSC to enhance their biological activities for higher efficiency in ischemic stroke treatment. Nonetheless, the exact mechanism underlying of such a natural product-based preconditioning strategy and its actual effectiveness in repairing the ischemic area in the brain remained ambiguous. Therefore, transcriptomic analysis using microarrays presented in this study was imperative to reveal the key genes, pathways and biological activities involved in the neurogenesis and brain tissue remodeling after the BEF-preconditioned NSCs ischemic stroke treatment for the first time.

1.3 Significance of the study

Current ischemic stroke treatment is still very limited, and the rate of recurrence is also very high. Moreover, the high costs of access to post-stroke care and monitoring at private health care settings is also a difficult challenge for stroke survivors. Therefore, the application of potential neuroprotective BEF extracted from *O. indicum* medicinal plant to precondition rat NSCs could provide alternative ischemic stroke treatment with higher efficiency at more affordable cost. This is because *O. indicum* plant is a native plant that can be easily grown locally for the source of the targeted baicalein flavonoid, making it accessible at low cost. Most importantly, the baicalein extracted from this plant has been proven non-toxic based on the Organization for Economic Co-operation and Development (OECD) Toxicity Test Guidelines for Chemicals (Othman *et al*., 2023), making it safe to be applied in clinical setting in future. Moreover, this study also provided a detailed insight into the assessment of neurological recovery after the transplantation of BEF-preconditioned NSCs to the infarcted area compared to non-preconditioned NSCs treatment group. Subsequently, this study also elucidated transcriptomic profile to reveal key genes and pathways underlying ischemic stroke

recovery mechanisms induced by BEF-preconditioned NSCs transplantation into ischemic stroke rat models. Understanding of such mechanisms will be essential to pioneer the establishment of NSC-based regenerative therapy for ischemic stroke in clinical setting in future.

1.4 Objective of the study

1.4.1 General objective

To elucidate the key regulator genes and pathways underlying ischemic stroke recovery mechanisms induced by neural stem cell preconditioned with baicalein-enriched fraction of *O. indicum* transplantation in ET-1 ischemic stroke rat models.

1.4.2 Specific objectives

1. To study the physicochemical features of *O. indicum* plant, and extract BEF from the leaves of *O. indicum* plant.
2. To determine the cytotoxicity effects and the optimum concentration of BEF for NSC preconditioning *in vitro.*
3. To determine the effects of BEF-preconditioned NSC transplantation on neurological functions and behaviors of the ET-1 ischemic stroke rat model.
4. To identify the key genes and pathways involved in the regulation of neurological functions and behaviors recovery in the ET-1 ischemic stroke rat model.

1.5 Hypothesis of the study

1. Physicochemical features of *O. indicum* could be successfully determined prior to extraction and fractionation of baicalein in the form of BEF.
2. BEF could precondition NSCs and activate cell proliferation rate at optimum concentration and duration.
3. BEF-preconditioned NSC transplantation could significantly improve the neurological deficits in ET-1 ischemic stroke rat models compared to the non-treated or non-preconditioned NSC-treated groups.
4. Transcriptomic analysis using microarray assay could successfully reveal the key genes and pathways involved in the improvement of ischemic stroke deficits triggered by BEF-preconditioned NSC transplantation in ET-1 ischemic stroke rat models.

CHAPTER 2

LITERATURE REVIEW

2.1 Stroke

Stroke is known as cerebrovascular disease (CVA) caused by either blocking an artery or bursting a blood vessel in the brain. It is one of the leading causes of death and disability in many countries, including Malaysia. Its incidence in Malaysia has steadily increased over the last 2 decades. In 2019, data from Malaysia recorded 47, 911 incident cases, 19,928 deaths, 443,995 prevalent cases and 512,726 disability-adjusted life years (DALYs) lost due to stroke (Tan & Venketasubramanian, 2022).

There are two main classes of stroke: ischemic stroke and hemorrhagic stroke. According to Clinical Practice Guidelines in Malaysia, the majority of stroke cases in Malaysia are ischemic stroke (~72%), while only approximately 18% of strokes cases are hemorrhagic stroke. Acute ischemic stroke is continued to be the major cause of morbidity and is currently the third leading cause of mortality in Malaysia (Clinical Practice Guidelines Management of Ischemic Stroke, 2021). Therefore, this study focused on the investigation of treatments for ischemic stroke.

2.1.1 Ischemic stroke

Ischemic stroke is triggered by the interruption of blood supply to a part of the brain, leading to oxygen and nutrient deprivation in the affected brain area and subsequently causing irreversible brain cell death. Ischemic brain cell death resulted in brain morphological alterations, neurological behavioral disturbance, and significant impairment of cognitive functions (Heiss, 2016).

There are three major types of ischemic stroke, namely thrombotic stroke, embolic stroke and lacunar stroke (Figure 2.1). Thrombotic stroke occurs when a thrombus (blood clot) develops in the brain arteries and disrupts normal blood flow to the brain tissue (Figure 2.1a). Most of the thrombus develops due to atherosclerosis which is a deposit of a fatty substance called 'plaque' on the artery lining (Maida *et al.*, 2020). The thrombus can halt the flow of blood and form blood clots that totally occlude the blood flow (Rojsanga *et al.*, 2019).

On the other hand, embolic stroke is caused by an embolus that has formed in other body parts (e.g., heart or carotid arteries) and travels via the bloodstream of internal carotid artery until it reaches a smaller blood vessel in the brain, where its passage is blocked (Figure 2.1b). It accounts for approximately 14-30% of the total ischemic stroke incidences (Maida *et al.*, 2020). Embolic stroke is usually associated with atrial fibrillation, such as abnormal heart rhythm in which the atria does not beat effectively and increases the risk of clot formation. It can also be caused by a clot dislodging from the atherosclerotic plaque formed in the aorta and carotid artery (Yang *et al*., 2022).

Lastly, lacunar stroke occurs when an occlusion (clot) forms in a small lenticulostriate arteries branched from the main middle cerebral artery (MCA) in the brain that penetrates deep into the organ (Figure 2.1c). The lacunar stroke is often associated with chronic hypertension, itself facilitating a small arteriole to become abnormal and susceptible to occlusion from micro-thrombi (Regenhardt *et al.,* 2018). This subtype is recorded to has 15-25% of all cerebral ischemic infarctions (Maida *et al*., 2020).

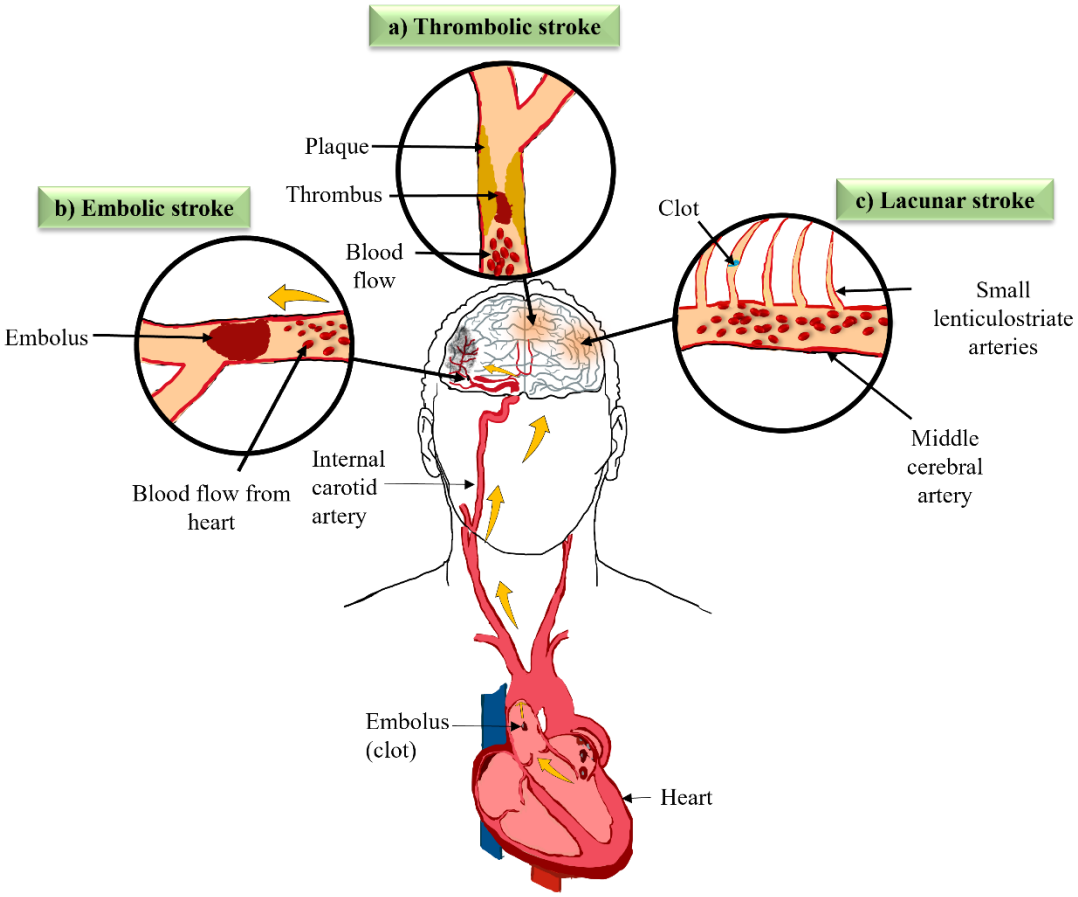

Figure 2.1 Illustration of three major types of ischemic stroke (a) thrombotic, (b) embolic and (c) lacunar stroke. (modified from: (*Lacunar Stroke Guide: Causes, Symptoms and Treatment Options*, 2023)).

2.1.2 Pathophysiological events of ischemic stroke

The occlusion of a cerebral infarction resulted in rapid depletion of oxygen and energy supplies and subsequently triggered a cascade of pathophysiological events including excitotoxicity, oxidative stress, mitochondrial impairment, blood-brain barrier dysfunction, inflammatory response and eventually caused neuronal cell death as illustrated in Figure 2.2.

Excitotoxicity is a hallmark sign of ischemic stroke onset. It occurs due to the deterioration of membrane ion gradient, excessive calcium influx, and release of excess glutamate after the shutdown of oxygen and nutrients supplies (Kumar *et al.*, 2019). This excitotoxicity could directly contribute to inflammatory responses and subsequently lead to the breakdown of blood-brain barrier (BBB) by disrupting the tight junctions between endothelial cells, allowing the entry of substances that would normally be restricted and potentially leading to further damage in the brain (Chen & Li, 2021). Additionally, excessive intracellular calcium and glutamate lead to cell oxidative stress. Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses that can relate to tissue damage. ROS are highly reactive molecules with one or more unpaired electrons. It can cause varying damage and dysfunction of cells in which the free radicals can react with DNA, proteins and lipid at cell membranes by stealing their electrons through oxidation process following reperfusion of ischemic tissue (Arman, 2019). This oxidation stress causes further tissue damage and triggers apoptosis after ischemic stroke.

In the meantime, cell apoptosis could impair the BBB which play critical role as a tight diffusion barrier to strictly regulate the movement of molecules, ions and cells between the CNS and the blood system (Dong, 2018). Once BBB is impaired, it could

result in leakage of circulating neurotoxic substances into the CNS, causing inflammatory responses such as intravascular leukocytes activation and release of proinflammatory mediators from the ischemic endothelium and brain parenchymal, thus increased the chances of brain tissue injury which could lead to cerebral damage (Kumar *et al.*, 2019). All these events are interrelated and coordinated which can ultimately lead to ischemic necrosis in the severely infarcted ischemic region. Ischemic necrosis is an uncontrolled cell death and morphologically characterized by cellular and organelle swelling, leads to disruption of the nuclear, lysis of cell and spillage of intracellular contents into surrounding tissue and eventually leading to irreversible tissue damage (Khalid & Azimpouran, 2022).

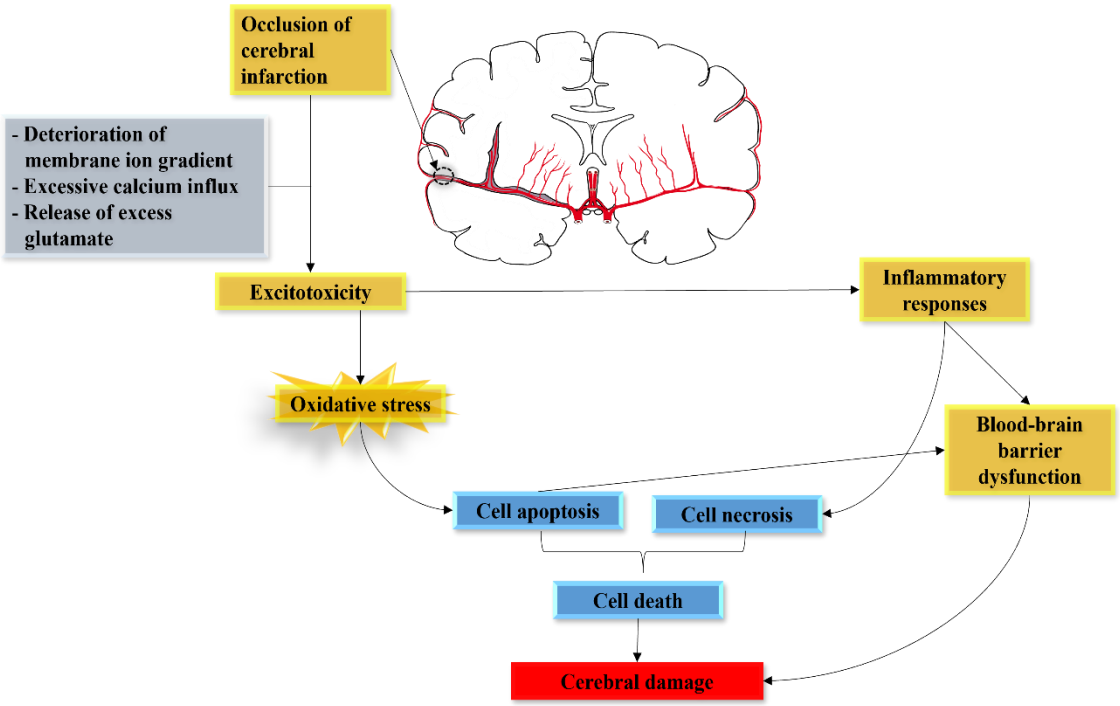

Figure 2.2 Pathophysiology of ischemic stroke leads to cerebral damage, initiating a complex series of events. Excitotoxicity gives rise to oxidative stress and a post-ischemic inflammatory response leading to cell apoptosis, cell necrosis, blood-brain barrier dysfunction. Ultimately, these processes culminate in led cell death and consequently, cerebral damage. (modified from: (Mir *et al*., 2014)).

2.1.3 Risk factors of ischemic stroke

The understanding of the risk factors for ischemic stroke among Malaysian opens a passage for primary prevention strategies specifically according to the population needs. Most of the risk factors of ischemic strokes are similar, even though there are some differences among the etiologic categories of ischemic stroke. Generally, ischemic stroke risk factors can be grouped into two, namely non-modifiable and modifiable risk factors. The non-modifiable risk factors are age, sex and family history. Generally, stroke is a disease of aging that has been recorded with doubling incidence rates after age of 55 years. The ischemic stroke incidence and mortality vary by sex and age, in which the incidence and mortality are similar in both sexes among young patients (below 45 years of age). Between age 45 and 74 years, males show higher incidence

and mortality than woman. However, the incidence likely to be reversed at age of 75 years, where women are mostly at higher risk for ischemic stroke (Wajngarten & Silva, 2019).

On the other hand, modifiable risk factors including hypertension (67%), diabetes mellitus (39.6%), smoking (25.2%), hypercholesterolemia (23%) and atrial fibrillation (3%) according to data obtained from Malaysia National Stroke Registry (Feigin *et al.*, 2021). The order of these risk factors remained fairly the same for five years period of observation. Hypertension is always the predominant risk factors for stroke attack, mainly because the stress on the blood vessels (vascular stress) associated with high blood pressure can exacerbate changes in blood vessel which leads to a larger causal factor for stroke. When the blood vessels experience chronic stress due to hypertension, it can lead to changes in the structure and function of the BBB of the brain and can further compromise the barrier's ability to control the passage of substances (Elsaid *et al.*, 2021).

Moreover, a major contributor to coronary heart disease that can complicate ischemic stroke is hyperlipidemia, which is also known as hypercholesterolemia. It is caused by excessive consumption of a high-cholesterol diet leading to high levels of blood lipids that increase the risk of stroke incidence. Hyperlipidemia has been reported to increase BBB permeability and promote brain edema formation in cerebral ischemia in the acute phase (Menet *et al.*, 2018).

Lastly, atrial fibrillation is also an important risk factor for stroke in which it comes up to 15% of all strokes worldwide and produces more severe disability and higher mortality compared to non-atrial fibrillation-related strokes. Its consequence was caused by decreased oxygen blood flow in the left atrium and thus led to embolism in the brain (Kuriakose & Xiao, 2020).

2.2 Current treatment for ischemic stroke

Following a blood vessel occlusion, brain cells can deteriorate rapidly and ultimately die within minutes. Due to the acute onset, repairing the cellular damage in the ischemic brain remains one of the most crucial challenges of medical science. Therefore, up to date, there are still limited treatments available for patients with acute ischemic.

2.2.1 Thrombolysis

Application of intravenous thrombolysis with rt-PA shows a significantly reduced mortality rate and disability associated with ischemic stroke within 4.5 h after onset and later treatment may improve outcomes in selected patients up to 9 h after stroke. To date, rt-PA is the one and only approved drug for ischemic stroke treatment by FDA. However, a major limitation of this pharmacological treatment is that rt-PA drug must be administered to the patient within a limited time window typically 3 to 4.5 hours of stroke onset to be effective (Urrutia *et al*., 2018). This short therapeutic window has greatly limited its utilization rates in routine clinical practice because hospitalization delays for stroke occurrence is very common (Mohamadpour *et al.,* 2019). Thus, the administration of rt-PA within a specific hour such as 4.5 h after onset of stroke is difficult in many situations. Over the past years, it was found that almost 80% of ischemic stroke patients were late to be admitted to hospital within the golden treatment window of rt-PA due to several factors including slow or gradual onset, mild neurological symptoms, patients were alone during the onset or those who were unbale to contact anybody when their symptoms occurred (Mohamadpour *et al.*, 2019). Any administration of rt-PA beyond the golden therapeutic window could result in severe

side effects, commonly being hemorrhage or even life-threatening (Liaw & Liebeskind, 2020).

2.2.2 Mechanical thrombectomy

Mechanical thrombectomy (MT) is an available treatment with high efficacy within 6-8 h of ischemic stroke onset. Instead of using thrombolytic agents, MT technique physically remove blood clot using stent-retriever devices and it is most recommended in cases of large proximal cases vessel occlusion, in which thrombolysis agent is less than 10% effective (Derex & Cho, 2017). Nonetheless, MT treatment option is limited by the technical difficulty to navigate stent-retriever wire in delicate intracranial vessels to reach the occluded site. In some cases, the MT surgical procedure could cause trauma to vessels, distal embolization, vessel dissection and vasopasm leading to worsening of stroke (Hurd *et al.,* 2021). Moreover, both thrombolysis and MT have critical problem in which they only able to regenerate the dead neuronal cell to restore brain function completely (Samaniego *et al.*, 2018). Due to these reasons, alternative treatments based on regenerative cell-based therapy have been discovered to address these problems.

2.3 Regenerative treatment for ischemic stroke

Over the past decades, stem cell-based therapy has been discovered as an alternative treatment for ischemic stroke due to the ability of stem cells to regenerate the infarcted area. There are two fundamental characteristics of stem cells that make them beneficial in ischemic stroke therapy. First, stem cells are able to self-renew and differentiate into viable neuronal cells to replace the dead cells after ischemic stroke attack (replacement mechanism). Secondly, stem cells are able to secrete neuroprotective cytokines to maintain viable microenvironment (paracrine mechanism)

(Samaniego *et al*., 2018). Many types of stem cells have been studied for their regenerative potential in treating neurological diseases including the mesenchymal stem cells (MSCs) (Abdullahi *et al.*, 2021), induced pluripotent stem cells (iPSCs) (Salikhova *et al.,* 2021), dental pulp stem cells (DPSCs) (Zhang *et al*., 2018) and as well as NSCs (Hamblin & Lee, 2021).

Among the different stem cell types extensively studied nowadays, NSCs are the only stem cells found in the brain. The NSCs are originally in the neuronal lineage, and they possess the natural instinct to differentiate into neurons, astrocytes and oligodendrocytes. Furthermore, NSCs also can preserve the BBB, ameliorate inflammation, promote neurogenesis and angiogenesis and promote functional recovery and motor skills (Boese *et al.*, 2018). Hence, they have very high potential for ischemic stroke treatment. In this study, the therapeutic potential of NSC for ischemic stroke treatment was investigated.

2.3.1 Neural stem cells (NSCs)

Neural stem cells (NSCs) are multipotent cells that generate progenitor cells, which later primarily differentiate into neurons, astrocytes and oligodendrocytes to maintain brain homeostasis (Łos *et al.*, 2018). They were first isolated from the lateral ventricle ependymal cell layer called the subventricular zone (SVZ) in the mouse brain by Reynold and Weiss (1992). NSCs possess the unique ability to mitigate stroke pathology since they are able to migrate through the CNS and repopulate lesion site after ischemic injury (Baker *et al*., 2019).

There have been studies reporting natural endogenous neurogenesis in the ischemic penumbra region of the brain (Guo *et al*., 2022; Hamblin & Lee, 2021; Tornero, 2022). Kevin *et al*. also reported that tissue after stroke attack was replaced by

new viable immature neuronal cells (neuroblasts) which were differentiated from endogenous NSCs originated from SVZ. However, the natural neurogenesis of endogenous NSCs is insufficient and is not enough to restore the damaged area and whole brain functions (Hurd *et al*., 2021). Consequently, the efficiency of endogenous NSC-based therapy is not fully achieved. This could be due to hostile microenvironment in the ischemic regions which limit the survival rate of these NSCs (Hicks *et al*., 2009; Othman & Tan, 2020).

Therefore, in this study, the therapeutic potential of NSCs was enhanced using natural product-based preconditioning strategy. This approach was an attractive option due to its potential to increase the stem cell viability, survival, proliferation and migration after transplantation to ischemic area (Othman & Tan, 2020).

2.4 Preconditioning strategies to enhance NSC-based therapy

Various preconditioning strategies have been utilized and explored in this recent years to improve the donor cell survival after transplantation and to enhance their therapeutic potential by improving their paracrine effects, and boosting their capacity for tissue regeneration and for the cerebral function restoring (Hu & Li, 2018).

Many different preconditioning strategies have been tested in stem cells prior to cell grafting. Among them, hypoxic preconditioning is by far the most commonly applied strategies to enhance stem cells for ischemic disease therapy. In general, hypoxia is characterized as insufficient oxygens delivery to tissues and cells in the body and is prevalent in many human physiology processes and diseases. Hypoxic preconditioning involves brief hypoxia (~1-5% oxygen level) exposure periods in various cell types, organs, animal model and humans, leading to upregulation of genes associates with cell stress response pathways, protective signaling pathways, and

enhance sturdiness to ischemia via the activation of a master transcription factor known as hypoxia-inducible factor (HIF) (Othman & Tan, 2020). Nonetheless, hypoxic preconditioning for stem cell culture is technically challenging due to the difficulty to monitor intermittent exposure to ambient air oxygen level (~21%) during cell culture procedure in laboratory (Al-Ani *et al*., 2018).

On the other hand, natural compounds with neuroprotective effects are another promising strategy to briefly expose the stem cells following their properties in a more sustainable and environmentally friendly strategy. This pharmacological preconditioning represents a novel and successful technique to stimulate stem cells for the secretory of beneficial cytokines for regenerative treatment. Shu *et al.* (2022) reported that bone-marrow-derived mesenchymal stem cell (BMSC) preconditioned with trehalose (Tre), a pharmacological agent was successfully increased proliferation, enhanced the survival of transplanted stem cells under oxidative stress of ischemic nerve injury and ultimately improved functional recovery (Shu *et al*., 2022). Furthermore, Lee *et al*. 2020 also proposed strategies to enhance biological processes of MSCs, including their migration and proliferation. The strategy involved preconditioning MSCs with ethionamide, an antibiotic compound, and it was found that these cells survived longer in the brain after transplantation, thereby augmenting their therapeutic properties through the secretion beneficial paracrine factors in neurodegenerative diseases (Lee *et al.*, 2020). In this study, NSCs were preconditioned with a neuroprotective natural compound known as, baicalein, extracted from the leaves of *O. indicum* plant.

2.5 *Oroxylum indicum* plant

O. indicum is a genus of medium-sized deciduous tree belongs to the Family Bignoniaceae. This is characterized by brown bark and large pinnate leaves and is mainly distributed in India, Sri Lanka, Malaysia, China, Thailand, Philippines, and Indonesia. The list of botanical classification of *O. indicum* was shown in Table 2.1.

Table 2.1 The classification of botanical for *O. indicum.*

Botanical classification	
Kingdom	*Plantae*
Class	*Magnoliophyta*
Order	*Lamiales*
Family	*Bignoniaceae*
Genus	*Oroxylum*
Species	*Indicum*

Figure 2.3 illustrates the *O. indicum* plant which can grow up to 20 meters of height with fruits hanging down from the bare branches like dangling swords and the flowers bloom on top of the tree (Figure 2.3(a)). The bark of the plant is light brown or greyish-brown colored, which is soft and spongy with numerous corky lenticels (Figure 2.3(b)), while the leaves are pinnately compound which normally grows up to 3-10 cm long and the adjacent leaflets of the leafstalks are 6-15 mm long (Figure 2.3(c)). The flower corolla is funnel shaped with 5 lobes and 10 cm long, subequal with wrinkled margin, outside is reddish, while inside is yellowish to pinkish colored (Figure 2.3(d)). The plant flowers are numerous in numbers, bisexual and the stalks of the flowers is roughly 30 cm. The fruits are winged, woody, large and flat, capsule or sword shaped (Figure 2.3(e)). During rainy season in December until March, the flowers are usually

blooming, and fruit appears (Jagetia, 2021; Kalra & Kaushik, 2017; Salleh *et al*., 2020). Moreover, this plant also has mutualistic symbiosis with an actinomycete *Pseudonocardia oroxyli,* a gram-positive bacterium that has the capability to produce many secondary metabolites exhibiting a wide variety of biological activities (Ahad *et al.,* 2012; Lalrinzuali *et al.,* 2018*). O. indicum* is known for its high commercial and economic importance with several medicinal properties as stated in the Ayurveda and Unani system of medicines. It has been found to have astringent, antioxidant, anti-inflammatory, antihelminthic, antibronchitic, antileucodermatic, antirheumatic, anti-anorexic and many other properties of medicinal (Pondugula *et al*., 2021).

Figure 2.3 Representative images of *O. indicum* plant and its components; a) *O. indicum* plant, b) bark, c) leaves, d) flower and e) sword shape of fruit

2.5.1 Phytochemical of *O. indicum*

To date, many secondary metabolites such as alkaloids, flavonoids, tannins, terpenoids, carotenoids and anthocyanin have been reported from different parts of *O. indicum* with flavonoids as the major storage constituent (Amancharla, 2017). Major flavonoid constituents present in *O. indicum* are baicalin, baicalein, scutellarin, oroxylin -A and chrysin (Amancharla, 2017; Rojsanga *et al*., 2020). Based on phytochemical analysis done by Amancharla, 2017 on different parts of *O. indicum*, the presence of flavonoids was strongly positive in the fresh leaves extract followed by the fruits and seeds. In addition, leaves part have been focused in this study due to their availability are evergreen for every season compared to other parts, flowers and fruits (Amancharla, 2017). Commonly found compounds from each part of *O. indicum* are stated in Table 2.2.

Table 2.2 Different parts of *O. indicum* and the compounds.

Plant part	General name of active compound found	References
Leaves	Flavonoids: baicalein, chrysin, oroxylin, Phenols: stilbenes, coumarins, Alkaloids: campothecin, vinblastine, vincristine, Tannins, Glycosides, Quinones: blumbagin, shikonin, thymoquinones	(Rojsanga *et al.,* 2017)
Fruits	Flavonoids: baicalein, chrysin, oroxylin, Triterpene carboxylic acid, Quinones: blumbagin, shikonin, thymoquinones.	(Sithisarn *et al.*, 2016)
Seeds	Flavonoids: baicalein, baicalein-7-O-diglucoside, baicalein-7-O-glucoside, chrysin, oroxylin A, oroxylin B, apigenin, Alkaloids: campothecin, vinblastine, Terpenes, Saponins, Oils.	(Samatha *et al.*, 2012)
Root barks	Flavonoids: baicalein, chrysin, oroxylin-A, scutellarin-7-rutinoside, sitosterol, galactose, biochanin-A, Alkaloids: campothecin, vinblastin, vincristin, Ellagic acid, Biochanin-A.	(Zaveri *et al.*, 2008)
Stem barks	Flavonoids: baicalein, baicalein-7-O-glucoside, baicalein-7-O-diglucoside, chrysin, Phenols: stilbenes and coumarins.	(Lalrinzuali *et al.*, 2018)

2.6 Baicalein

Baicalein (5,6,7-trihydroxyflavone) is composed of 15 carbon compounds ($C_{15}H_{10}O_5$) as illustrated in Figure 2.4. It is also often identified as a drug for liver protection and reduces inflammatory diseases (Shi *et al.*, 2017). Various other biological functions of baicalein also have been revealed, such as baicalein could significantly restrain 12-lipoxygenase (Li *et al.*, 2019), exert antioxidant and anti-inflammatory effects (Dai e*t al.*, 2017; Shi *et al*., 2018), involve in autophagy activation

(Wu *et al*., 2018), activate cancer cell apoptosis (Liu *et al.*, 2017) and attenuate cancer cell proliferation and invasion (Wang *et al*., 2015). Most importantly, baicalein was found to possess neuroprotective effects and could enhance neuronal cell proliferation (Liang *et al*., 2017). Therefore, baicalein extracted from the *O. indicum* is postulated to be able to enhance the therapeutic potential of NSC for ischemic stroke therapy in this study.

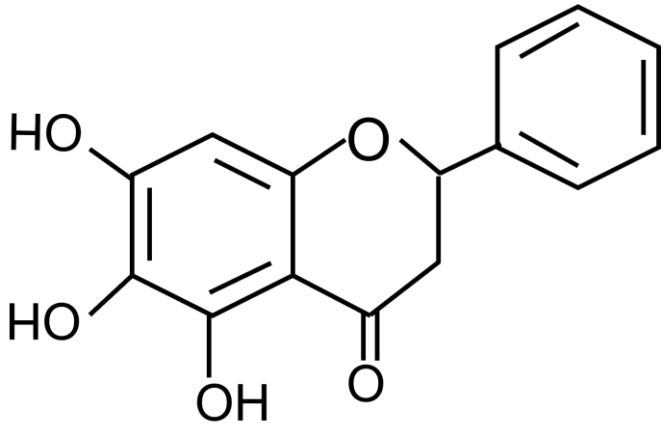

Figure 2.4 The chemical structure of baicalein, $C_{15}H_{10}O_5$.

2.7 Endothelin-1 ischemic stroke rat model

In this study, NSCs preconditioned with baicalein extracted from *O. indicum* were transplanted into *in vivo* ischemic stroke rat models induced using a vasoconstrictor known as ET-1. ET-1 is 21-amino acid peptide produced by vascular endothelium and it has potent vasoconstrictor action first identified by Yanagisawa's group in 1988 (Yanagisawa *et al*., 1988). In addition, it also possesses long-acting vasoconstriction properties which act through two types of receptors, the endothelin A and endothelin B receptors which are responsible for potent vascular contraction, cell proliferation and a proinflammatory effects (Kowalczyk *et al*., 2015).

Up to date, the application of ET-1 is the only method to induce stroke in conscious animals (Abeysinghe & Roulston, 2018). This is because the ET-1 can be