THERAPEUTIC POTENTIAL OF NEURAL STEM CELLS PRECONDITIONED WITH BAICALEIN-ENRICHED FRACTION IN ISCHEMIC STROKE RAT MODEL

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

µg	microgram
µl	microlitre
µm	micrometre
ANOVA	one-way analysis of variance
ARASC	Animal Research and Service Centre
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
BBB	blood-brain barrier
BDNF-pCREB	brain-derived neurotrophic factor-phosphorylated cathelicidin antimicrobial peptide (cAMP) response element-binding
BEF	Baicalein-enriched fraction
bFGF	recombinant human basic fibroblast growth factor
CNS	central nervous system
CO^2	carbon dioxide
CT	computed tomography
CVA	cerebrovascular accident
DALY	disability-adjusted life years
DG	dentate gyrus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
D-PBS	Dulbecco phosphate buffer saline
DPPH	2,2-diphenyl-1-picrylhydrazyl
EGF	recombinant human Epidermal Growth Factor
ELISA	Enzyme-linked immuno-absorbent assay
	and others
ET-1	endothelin-1
FDA	Food and Drug Administration
g	gram
GSH-Px	plasma glutathione peroxidase
H&E	haematoxylin and eosin

H_2O_2	hydrogen peroxide
HPLC	high-performance liquid chromatography
IACUC	Institutional Animal Care and Use Committee
IL-1β	interleukin-1-beta
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IVF	*in vitro* fertilization
kg	kilogram
MAO	monoamine oxidase
MCA	Middle cerebral artery
MCAo	middle cerebral artery occlusion
mg	miligram
mL	millilitre
mNSS	modified neurological severity score
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n.d.	not dated
NaCl	sodium chloride
NF-kB	nuclear factor kappa B
NIHSS	National Institutes of Health Stroke Scale
NO	nitric oxide
NSC	Neural stem cell
O. indicum	*Oroxylum indicum*
OD	optical density
PBS	Phosphate Buffer Saline
PBS	phosphate buffer saline
PI3K/Akt	phosphoinositide-3-kinase/Akt
PMNs	polymorphonuclear neutrophils
PTEN	phosphatase and tensin homolog deleted on chromosome
ROS	reactive oxygen species
SD	Sprague Dawley

SEM	Standard Error of Mean
SGZ	subgranular zone
SOD	superoxide dismutase
SSS	Scandinavian Stroke Scale
SVZ	subventricular zone
TLC	thin layer chromatography
TNF- α	tumor necrosis factor alpha
tPA	tissue plasminogen activator
TTC	2,3,5-triphenyltetrazolium hydrochloride
UV	ultraviolet
v/v	volume/volume
VEGF	vascular endothelial growth factor
w/v	weight/volume
WHO	World Health Organization

LIST OF APPENDICES

POTENSI TERAPEUTIK SEL STEM SARAF YANG DIPREKONDISI DENGAN FRAKSI-KAYA BAIKALEIN DALAM MODEL TIKUS STROK ISKEMIA

ABSTRAK

Strok iskemia, yang dicetus oleh gangguan aliran darah di dalam otak secara tiba-tiba, boleh menyebabkan kematian sel neuron. Sejak kebelakangan ini, cantuman sel stem saraf (NSC) multipoten telah muncul sebagai terapi yang berpotensi untuk menjana semula tisu otak yang rosak. Walau bagaimanapun, persekitaran mikro yang tidak sesuai di kawasan otak iskemia mengancam ketahanan hidup sel yang dipindahkan. Oleh yang demikian, kultur NSC dioptimumkan dengan baikalein, sebatian aktif pelindung saraf yang diekstrak dari tumbuhan *Oroxylum indicum* untuk meningkatkan kadar kelangsungan hidup NSC setelah pemindahan ke otak iskemia. Baikalein merupakan salah satu flavonoid utama didalam *O. indicum* yang dilaporkan mempunyai kesan pelindung saraf. Di dalam penyelidikan ini, *BEF* berjaya diekstrak dari daun *O. indicum* dan dinilai menggunakan HPLC dan keupayaan menghapuskan radikal bebas dibandingkan dengan baikalein sintetik sebagai kontrol positif. Model tikus strok iskemia dihasilkan menggunakan endothelin-1 (ET-1) yang menyekat arteri serebrum tengah (MCA) dan menyebabkan kerosakan iskemia di otak. NSC yang telah dikultur secara *in vitro* telah dikondisikan dengan BEF pada dos optimum 3.125 µg/ml selama 48 jam yang ditentukan melalui ujian MTT sebelum sel-sel ditransplantasikan kepada kumpulan tikus strok iskemia. Tingkah laku tikus dan keparahan strok telah diperhatikan dan direkodkan selama 14 hari. Peningkatan dalam tingkah laku strok berlaku dalam masa 14 hari selepas pemindahan NSC yang dikondisikan oleh BEF berbanding dengan kumpulan pemindahan NSC tanpa dikondisi. Melalui pewarnaan TTC, kumpulan yang dirawat dengan NSC yang dikondisi dengan BEF menunjukkan

pengurangan infarksi otak yang sangat signifikan (11.535 ± 1.44%), berbanding kumpulan yang dirawat dengan NSC tanpa dikondisi (17.784 ± 2.33%) dan kumpulan yang tidak dirawat (23.807 ± 2.60%). Tambahan pula, kumpulan yang dirawat dengan NSC yang dikondisi dengan BEF juga menyebabkan *angiogenesis* yang signifikan dan pengurangan dalam degradasi neuron, nekrosis sel dan keradangan, berbanding kumpulan lain. Sebagai kesimpulan, kajian ini telah membuktikan potensi BEF yang diekstrak dari *O. indicum* dalam menyumbang kepada kelangsungan hidup dan proliferasi NSC dan meningkatkan potensi terapeutik NSC dalam membaik-pulihkan tisu neuron yang rosak pada model tikus strok iskemia .

THERAPEUTIC POTENTIAL OF NEURAL STEM CELLS PRECONDITIONED WITH BAICALEIN-ENRICHED FRACTION IN ISCHEMIC STROKE RAT MODEL

ABSTRACT

Ischemic stroke, triggered by the abrupt interruption of cerebrovascular blood flow, could lead to permanent neuronal cell death. Recently, multipotent neural stem cell (NSC) grafting has emerged as potential therapy to regenerate the damaged brain tissue. However, the hostile microenvironment in the ischemic brain region is challenging for the survival of transplanted cells. In this regards, NSC culture was optimized with baicalein-enriched fraction (BEF) from *Oroxylum indicum*- to enhance the NSC survival rate after transplantation into ischemic brain. Baicalein is one of the major flavonoids present in *O. indicum* and has been reported to have neuroprotective effects. In this study, BEF was successfully fractionated from the leaves of *O. indicum* and quantified using HPLC and its radical scavenging activity was compared to synthetic baicalein as positive control. Ischemic stroke rat model was established using endothelin-1 (ET-1) which constrict the middle cerebral artery (MCA) to induce ischemic damage in the brain. *In vitro* expandable NSCs were preconditioned with BEF at optimum dosage of 3.125 µg/ml for 48 hours as determined through MTT assay before the cells were transplanted into the ET-1 induced ischemic stroke rat groups. Rat behaviours and stroke severity were observed and recorded for 14 days. Improvements in stroke behaviours occurred within 14 days after the transplantation of BEF-preconditioned NSC compared to non-preconditioned NSC transplantation group. Through TTC staining, the BEF-preconditioned NSC-treated group showed significant reduced brain infarct (11.535 ± 1.44%), compared to non-preconditioned NSC-treated group (17.784 ± 2.33%) and non-treated group (23.807 ± 2.60%). In

addition, BEF-preconditioned NSC-treated group also significantly reduced neuronal degradation and inflammation, while also increased blood vessel density compared to the other groups. As a conclusion, this study proved the potential of BEF extracted from *O. indicum* in contributing to the upregulation of NSC proliferation- and significantly improved the therapeutic potential of NSCs to repair damaged neuronal tissue- ischemic stroke rats.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Stroke, also known as cerebrovascular accident (CVA) or "brain attack", is one of the top five leading causes of death worldwide and the most frequent cause of adult disability among chronic diseases in developed countries (Phipps & Cronin, 2020; Salako & Imaezue, 2017). According to the statistics by *Dep. Stat. Malaysia* (2022), death caused by stroke or cerebrovascular diseases reached 6.5% of medically certified deaths in Malaysia. Stroke represents a serious health problem not only in Malaysia but also worldwide as it could cause irreversible neuronal damage to those who are afflicted (Zhang *et al.*, 2019). Stroke can happen to anyone; some people are at higher risk for different reasons such as age, family history, high blood pressure, smoking, being overweight, diabetes, and high cholesterol. Stroke recovery is usually a slow process and it depends on the severity of disease. Due to its high prevalence and long-term recovery process, stroke can be described as a devastating and distressing experience not only to the affected individual, but to their families or caretakers as well.

There are two types of strokes: ischemic stroke and haemorrhagic stroke. Ischemic stroke is the sudden death of brain cells due to lack of oxygen, caused by blockage of blood flow due to thrombosis or embolism, while haemorrhagic stroke happens when there is rupture of artery to the brain which causes bleeding to occur within the brain

(Kumar , 2016; Ruan & Yao, 2020). Both types of strokes can cause irreversible brain cell death, resulting in permanent brain damage. Stroke patients may suddenly experience paralysis, impaired speech, or loss of vision (Moskowitz *et al.*, 2010). Ischemic stroke is more common in Malaysia compared to haemorrhagic stroke (Loo & Gan, 2012); therefore, the present study focused more on the treatment for ischemic stroke.

Current ischemic stroke treatment is still very limited and the rate of recurrence is also very high (Zhang *et al.*, 2019). To date, only one drug has been approved by the United State Food and Drug Administration (FDA) for acute ischemic stroke treatment which is the tissue plasminogen activator (tPA) that helps to restore blood flow by dissolving the blood clots that cause ischemic strokes (Hamblin & Lee, 2021). Nonetheless, tPA is unable to regenerate dead neurons after stroke attack. Therefore, ischemic stroke patients still have to depend on rehabilitation to regain the loss of brain function. Some hospitals in Malaysia also provides alternative post-stroke therapies such as the traditional Malay massages 'urut Melayu' (Anuar *et al.*, 2010). However, the limited number of community-based rehabilitation centres to provide continuous support for stroke patients after discharge has resulted in stroke survivors being left to manage long term post-stroke impact on their own. 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. For those reasons alone, it is crucial to develop alternative therapeutic strategies for stroke with the purpose of elevating the life quality of stroke survivors.

Since the last few decades, stem cell grafting has become one of the most promising treatments of neurodegenerative diseases such as ischemic stroke (Locatelli *et al.*, 2009). This is mainly because stem cell has the ability to self-renew and differentiated into matured functional neuronal cells. These unique characteristics of stem cells make them a potential therapeutic agent for neuronal regenerative treatment. A study reported that brain tissue damaged after stroke could be replaced by endogenous neural stem cells (NSCs) (Hamblin & Lee, 2021). NSCs are multipotent stem cells that reside in subventricular zone (SVZ) and dentate gyrus (DG) of adult brain. NSCs can divide and differentiate into various brain cell types corresponding to their surrounding microenvironment in terms of their morphologies and functions (Golas, 2018). Endogenous NSC is also capable of directly restoring the damaged brain tissue and exert neuroprotection towards brain tissue during the acute phase of stroke. This corroborates the fact that NSC is a potential cell source in treating ischemic stroke.

However, effectiveness of *in vivo* NSC-based treatment is limited by low survival rate of transplanted NSCs in ischemic brain. Therefore, in this study, we proposed to precondition exogenous NSCs using baicalein, a flavonoid active compound presents in *Oroxylum indicum* plant which had been found to exert neuroprotective effect and increase cell proliferation rate (Liu *et al.*, 2017; Van Leyen *et al.*, 2006) before transplantation into ischemic stroke brains.

O. indicum is a medicinal plant that is consumed as ‘ulam’ by the locals as it possesses active compounds that related to wide range of biological and

pharmacological activities beneficial to human health (Kang *et al.*, 2019; Salleh *et al.*, 2020; Singh, 2015). It possesses hundreds of secondary metabolites such as flavonoid and the major constituent of flavonoid present in this plant is baicalein which is beneficial to human health. Numerous studies also showed that *O. indicum* is not toxic to humans and experimental animals even at high doses (Ahad *et al.*, 2012). Hence, the therapeutic potential of preconditioned NSCs using baicalein-enriched fraction (BEF) extracted from *O. indicum* plant on ischemic stroke rat model was studied.

In this research, ischemic stroke rat was induced with the injection of endothelin-1 (ET-1) which constrict the middle cerebral artery (MCA) that causes ischemic damage in the brain. Preconditioned-NSCs were introduced into the brain after stroke induction via the same cannula implanted for the injection of ET-1 therefore, justifying the reason that this procedure is less invasive than performing surgery on the rat model several times. The neurological behaviour changes and *ex vivo* histological examination on brain tissue of rat model were also elucidated to investigate the effects of BEF-preconditioned NSC treatment. As a summary, this study intensified the roles of baicalein extracted from the leaves of *O. indicum* in preconditioning NSC to enhance its therapeutic potential for ischemic stroke rat.

1.2 Problem statement

NSC-based treatment had been reported to have successfully increased neurogenesis *in vitro*. However, the turnover rate of resident NSCs is too slow to support regeneration because of their dormancy and the hostile environment inside the ischemic brain. Therefore, transplantation of exogenous NSCs was found to

significantly complement or replace damaged tissues (Chen *et al.*, 2016). Nonetheless, the effectiveness of exogenous NSC transplantation for ischemic stroke therapy is limited by the low retention rate of the cell after injected into an ischemic brain (Wei *et al.*, 2017). It was found that only a few grafted cells survived and successfully migrated from a small initial injection site to populate the target region for a prolonged duration in ischemic environment (Hicks *et al.*, 2009). As a result, NSC transplantation is still unable to effectively regenerate damaged tissues for complete recovery. In order to address this problem, preconditioning of stem cells prior to their transplantation is imperative to activate the stem cell survival signalling to counter the rigorous microenvironment after cell transplantation. This can significantly improve the cell survival after transplantation, and thus enhance its therapeutic potential for ischemic stroke treatment (Kang *et al.*, 2019). Therefore, in this study, we proposed to precondition exogenous NSCs using baicalein, a flavonoid active compound presents in *O. indicum* plant which had been found to exert neuroprotective effect and increase cell proliferation rate (Liu *et al.*, 2017; Van Leyen *et al.*, 2006) before transplantation into ischemic stroke brains.

1.3 Significance of the study

Preconditioning with neuroprotective natural product isolated from *O. indicum* plant is a promising technique to enhance the survival of NSCs against hostile environment in ischemic brain after transplantation. In addition, natural *O. indicum* plant is not toxic to human and experimental animal (Ahad *et al.*, 2012). Aside from that, this plant was chosen because it is easily grown locally- for the source of target baicalein flavonoid, making it accessible at low cost. Furthermore, the ET-1 ischemic

stroke rat model developed in this research is the only model that induces stroke without the need for anaesthesia thus functional assessments of rat model can be done while the rat is conscious, mimicking the clinical onset of stroke in human (Ansari *et al.*, 2013). Therefore, the findings of this study can become a reliable and promising strategy to apply natural product to enhance NSCs for ischemic stroke therapy.

1.4 Objectives of study

1.4.1 General objective

To study the therapeutic potential of neural stem cell (NSC)-based treatment for ischemic stroke using baicalein-enriched fraction (BEF) derived from the leaves of *O. indicum*.

1.4.2 Specific objectives

1. To determine the optimum BEF concentration and duration for rat NSC preconditioning *in vitro*.
2. To investigate the effects of BEF-preconditioned NSC treatment on ischemic stroke rats' neurological function
3. To investigate the effects of BEF-preconditioned NSC treatment on ischemic brain tissue morphology.

1.4 Hypothesis of the study

The hypotheses of the study are as follow:

1. BEF could precondition NSCs and activate the cell survival at optimum concentration and duration.
2. BEF-preconditioned NSC therapy helps to improve ischemic stroke rats' neurological function.
3. BEF-preconditioned NSC therapy helps to improve ischemic brain tissue morphology.

CHAPTER 2

LITERATURE REVIEW

2.1 Ischemic stroke

Stroke is the sudden death of brain cells due to lack of oxygen, caused by blockage or rupture of an artery which interferes with the blood flow to the brain. Stroke injury is thought to result from a cascade of events begin with energy depletion until irreversible cell death (Petty *et al.*, 2021). Stroke patients may suddenly experience paralysis, impaired speech, or loss of vision due to the interruption of oxygen to the brain (Moskowitz *et al.*, 2010). Stroke causes the greatest burden of disease worldwide caused by severe disability (Katan & Luft, 2018). Deficits of stroke include physical disabilities such as partial loss of motor function, sensory loss, language disorders, aphasia, visual disorders, and even memory loss (Pinter & Brainin, 2012).

There are two types of strokes, namely ischemic stroke and haemorrhagic stroke (Figure 2.1). Ischemic stroke is the sudden death of brain cells due to lack of oxygen, caused by blockage of blood flow due to thrombosis or embolism (Figure 2.1A) while haemorrhagic stroke is caused by haemorrhage or rupture of an artery to the brain which causes bleeding to accumulate and press on the adjacent parenchyma within the brain (Figure 2.1B). Both ischemic and haemorrhagic stroke are increasing in low to middle income populations due to the rising prevalence of stroke risk factors including diabetes, hypertension and atrial fibrillation (Khan *et al.*, 2017). However,

ischemic stroke accounts for more than 80% of strokes and is much more common in Malaysia and worldwide compared to haemorrhagic stroke (Jiao *et al.*, 2021; Loo & Gan, 2012; Petty *et al.*, 2021).

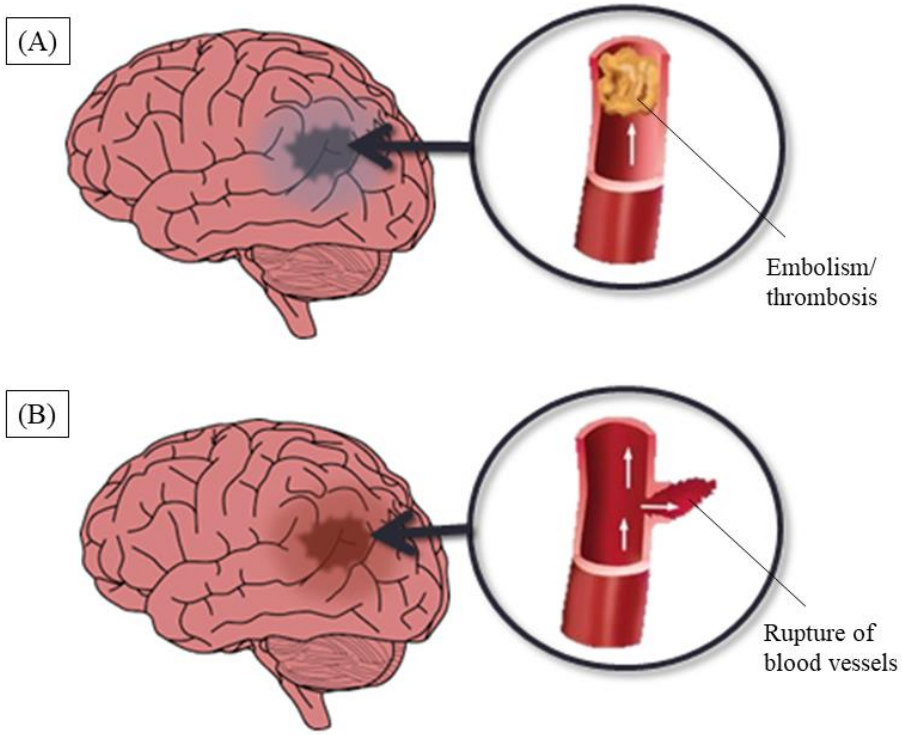

Figure 2.1 Type of stroke. (A) Ischemic stroke which is caused by occlusion of blood vessel to the brain and (B) haemorrhagic stroke which is caused by rupture of blood vessel leading to blood leakage.

2.1.1 Epidemiology of ischemic stroke

Between the year 2015 until 2020, Sierra Leone, North Korea, Mongolia, Cote D Ivoire, Yemen, Georgia and Indonesia are the countries with highest frequency of ischemic stroke (Muratova *et al.*, 2020). According to the latest data published in 2022, death due to ischemic stroke in Malaysia reached 6.5% of total deaths making it as one of the five principal causes of death in Malaysia (*Dep. Stat. Malaysia*, 2022). There were 47, 911 incident cases, 19,928 deaths, 443,995 prevalent cases, and 512,726

disability-adjusted life years (DALY) lost due to stroke in 2019 (Tan & Venketasubramanian, 2022). The distribution is highly affected by various risk factors including the non-modifiable hereditary factors such as family history or genetic predisposition, pre-existing comorbidities such as hypertension and dyslipidermia or modifiable lifestyle and environmental factors such as smoking or tobacco use, physical inactivity, and poor nutrition. 90% of all cases of stroke in the world are associated with modifiable risk factors (Muratova *et al.*, 2020), indicating that most of the stroke cases are avoidable.

The risk of recurrent is greatest during the first weeks or months after the initial stroke, and studies have shown that the risk increased from 3% to 22% in the first year to 53% within 5 years (Zheng & Yao, 2019). Age and dementia have been identified as the best predictors of recurrence (Appelros *et al.*, 2003). This is because older patients had a much higher rate of atrial fibrillation, hypertension, dyslipidemia and coronary artery disease which are constantly associated with ischemic stroke recurrence (Hillen *et al.*, 2003; Navis *et al.*, 2019; Zheng & Yao, 2019). On top of that, heart disease and heart failure also had been shown to have adverse influence on long-term prognosis of ischemic stroke in different ways making it difficult for fully recovery from ischemic stroke (Appelros *et al.*, 2003).

2.1.2 Initial diagnosis of ischemic stroke

Diagnosis of most of the ischemic stroke cases is straightforward based on the symptoms such as sudden numbness or weakness of the face, arm or leg, trouble speaking, sudden trouble seeing in one or both eyes, sudden dizziness, loss of balance

or coordination or sudden, severe headache with no known cause. However, for patients with unusual features such as gradual onset, seizure at the onset of symptoms, or impaired consciousness, the differential diagnosis should include migraine, postictal paresis, hypoglycaemia, conversion disorder, subdural hematoma, and brain tumors (Petty *et al.*, 2021).

In all patients with suspected ischemic stroke, computed tomography (CT) or magnetic resonance imaging (MRI) of the brain will be performed to acquire detailed brain imaging as quickly as possible, ideally within 20 minutes of the patient's arrival (Phipps & Cronin, 2020). Due to widespread availability and lower costs, CT scan is typically the first neuroimaging test performed in patients but MRI has a much higher sensitivity for acute ischemic changes. MRI imaging can identify brain ischemia within minutes of onset and is highly sensitive (88% sensitivity within 24 hours) and specific (95% specificity) for acute infarction (Mendelson & Prabhakaran, 2021).

2.1.3 Pathology of ischemic stroke

Cerebral circulation delivers continuous oxygenated blood, glucose and other nutrients which is vital in maintaining the viability and function of the brain. Moreover, the entire central nervous system (CNS) is highly sensitive to changes in oxygen concentration due to a high intrinsic oxygen consumption rate. Therefore, insufficient flow of blood due to vessel occlusion in ischemic stroke is unable to satisfy the requirement of oxygen and nourishing substances of the cerebral tissue within seconds to minutes after the loss of blood flow to a region of the brain (Hamblin & Lee, 2021). A cascade of pathophysiological events will occur following occlusion

involved during ischemic stroke such as neuronal degradation or cell death, tissue inflammation, apoptosis and necrosis resulting in tissue damage. These pathophysiological processes are interlinked, triggering each other in a positive feedback loop that terminates in neuronal destruction.

The energy deficit caused by the loss of neurons ability to synthesize adenosine triphosphate (ATP) is the main mechanism of cell death in the area of the cerebral infarction. This led to an inflammatory cascade, including oxidative stress, excitotoxicity, inflammatory cell infiltration, release of toxic inflammatory mediators, the slowing down of cell energy metabolism and depolarisation of the cell membrane. Gradual depolarisation of the cell membrane and loss of membrane potential increase the flow of sodium and allows for an osmotic transport of water to cells leading to the development of cytotoxic oedema. As a consequence, the accumulation of Na^+ and Ca^{2+} ions leads to organelle degeneration and loss of membrane integrity (Moskowitz *et al.*, 2010; Pawluk *et al.*, 2020). Brain and immune cells also produce reactive oxygen species (ROS), which not only causes primary vascular damage, but also triggers the development of the inflammatory response (Pawluk *et al.*, 2020). These responses will ultimately contribute to damage to tissues surrounding circulatory system, leading to further necrosis of the blood-brain barrier (BBB) and neurons, loss of neurovascular unit function, and disruption of the brain network (Jiao *et al.*, 2021; Maida *et al.*, 2020; Mo *et al.*, 2020).

Inflammation is widely regarded as a localised protective reaction, which helps restore balance when the body suffers from infection or tissue damage by killing pathogens and initiating the process of tissue repair (Biswanath Dinda *et al.*, 2017).

Inflammation in the brain accompanies the acute and chronic processes of central nervous system diseases and helps repair the damage of the nervous system under normal physiological conditions. However, the abnormal activation of inflammation will affect multiple signal pathways, inducing intense inflammatory cells infiltration in and around the injured brain tissue. Acquired brain injury such as ischemic stroke activate microglia along with activated astrocytes react via secreting cytokines resulting in an upregulation of cell adhesion molecules, allowing inflammatory cells to invade the brain parenchyma and secrete inflammatory mediators which lead to secondary brain injury, including brain oedema, haemorrhage and cell death (Kawabori & Yenari, 2015; Yuanyuan Li *et al.,* 2020). Experimental data have shown that resident microglia are activated within minutes of ischemia onset and produce a plethora of proinflammatory mediators which exacerbate tissue damage but may also protect the brain against ischemic and excitotoxic injury (Jin *et al.*, 2010). Occlusion to the MCA also causes fibrosis of the vascular cells and stimulates production of reactive oxygen species (ROS) and consequently leads to the development of oxidative stress. The vasoconstriction enhances the expression of adhesion molecules on vascular endothelial cells and stimulates the aggregation of polymorphonuclear neutrophils (PMNs) contributing to inflammation and endothelial dysfunction (Kowalczyk *et al.,* 2015). Although different mechanisms are involved in the pathogenesis of stroke, increasing evidence shows that ischemic injury and inflammation account for its pathogenic progression (Lakhan *et al.*, 2009).

Restoration of local blood perfusion in ischemic brain tissue plays a vital role in tissue repair and functional recovery after ischemic stroke. Studies have shown that stroke patients have reduced morbidity and longer survival time because of a higher

density of blood vessels (Yu *et al.*, 2014). The early response facilitates cerebral blood flow recovery through collateral circulation and anastomotic vessel activation, and the long-term response involves angiogenesis (Beck & Plate, 2009). Angiogenesis is the formation of new blood vessels from pre-existing vessels which helps improve tissue micro perfusion in ischemic boundary regions and reduce infarct volumes (Hui *et al.*, 2017). Angiogenesis may provide sufficient oxygen and nutrition for cerebral reconstruction and may participate in remodelling the damaged area to improve the recovery of the neural function of stroke patients (Carmeliet, 2000).

Ischemic infarct site can be defined by two distinct areas: the core and an outer stratum known as the penumbra (Figure 2.2). Initially after arterial occlusion, a central core of very low perfusion is surrounded by an area of dysfunction caused by metabolic and ionic disturbances yet the structural integrity is preserved. This surrounding area is known as the penumbra. The ischemic penumbra can be reinstated if cerebral blood flow is promptly restored. Therefore, in the first hours after ischemic stroke onset, the damage could be salvaged depending on the rate of reperfusion blood flow and the duration of ischemia. The timing is utterly important because the penumbra will eventually be incorporated into the infarct if reperfusion is not achieved (Petty *et al.*, 2021).

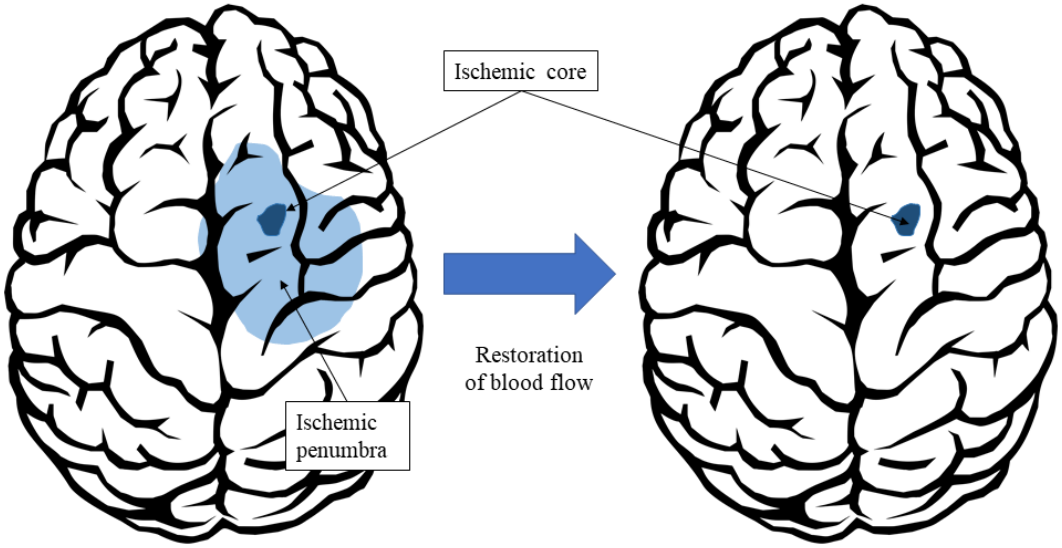

Figure 2.2 The ischemic core and penumbra. Ischemic core is the brain tissue that severely affected by ischemia and is destined to die while ischemic penumbra can be salvaged after restoration of blood flow. Adapted from (Petty *et al.*, 2021).

2.1.4 Current treatments for ischemic stroke and its limitations

Tissue plasminogen activator (tPA) is the only drug approved by the US Food and Drug Administration (FDA) for acute stroke treatment. tPA helps restore the blood flow in ischemic brain by dissolving the blood clots (thrombosis) in affected blood vessels following an ischemic stroke (Boese *et al.*, 2018). However, the major limitation is that it must be administered in a limited time window within 3 to 4.5 hours of stroke onset to be effective (Roth, 2011). This short therapeutic window has greatly limit its utilisation rates in routine clinical practice because hospitalization delays for stroke occurrence is very common (Yperzeele *et al.*, 2014). Over the years, it was found that almost 80% of ischemic stroke patients were late to be admitted to hospital within the golden treatment window of tPA due to several factors including slow or gradual onset, mild neurological symptoms or those who were alone and initially did

not contact anybody when their symptoms occurred (Jiao *et al.*, 2021; Wester *et al.*, 1999; Yperzeele *et al.*, 2014).

Besides tPA, another potential pharmacological drug for ischemic stroke known as acetylsalicylic acid (ASA), or aspirin is also used as secondary prevention medicine to reduce the risk of recurrent ischemic stroke. This is because aspirin acts as anticoagulant by thinning the blood and thereby prevents clots in vessels. However, the efficacy is limited because full anticoagulation could result in haemorrhagic transformation in the immediate poststroke period depending on the infarct size (Herpich & Rincon, 2020; Petty *et al.*, 2021). There are also side effects from the usage of ASA such as headache and general ill feeling or flu-like symptoms.

Surgical is another option to treat ischemic stroke. Clot can be removed through mechanical clot removal surgery within 24 hours after the onset of stroke symptoms. However, not all patients can go under the invasive surgical procedure especially if their carotid arteries are mostly blocked and aging factor. Moreover, surgery may increase the risk of bleeding or disability. Other than that, no other specific treatment is available to treat either focal cerebral ischemia or a global ischemic event (Tajiri *et al.*, 2013).

Some hospitals in Malaysia for example, Kepala Batas Hospital in Penang, Putrajaya Hospital in Federal Territory of Putrajaya and Sultan Ismail Hospital in Johore have traditional and complementary units that practice the traditional Malay massages or ‘urut Melayu’, which serve as an alternative post-stroke therapy (Anuar

et al., 2010). A non-profit organisation, the National Stroke Association of Malaysia also provides stroke rehabilitation services to enable stroke survivors to return to normal life as much as possible within the limits of their disabilities within Malaysia. However, the limited number of community-based rehabilitation centres to provide continuous support for people with stroke after discharge from hospital care has resulted in stroke survivors being left to manage long term post-stroke impact on their own. There is also difficulty to access to post-stroke care and monitoring at private health care settings due to the high costs.

Hence, it is crucial to develop alternative therapeutic strategies for stroke which primarily focused to help in full recovery and reduce the risk of deteriorating patient's quality of life during chronic phase rehabilitation. Since last few years, cell grafting has been the most encouraging approach for the treatment of neurodegenerative diseases and stroke (Locatelli *et al.*, 2009). Stem cell therapy is a novel treatment that exhibits potential for replacing current standard treatment for ischemic stroke. Neural stem cells (NSCs) are an ideal stem cell source that can be established to provide a continuous supply of neurons, astrocytes and oligodendrocytes to restore neural networks and vascular remodelling after ischemic injury (Annese *et al.*, 2017; Trounson & McDonald, 2015). In this study, the therapeutic potential of neural stem cell in treating ischemic stroke is further investigated.

2.2 Overview of stem cell

Stem cells are undifferentiated cells which have the ability to self-renew and develop into specific types of cells. Stem cells can be obtained from two main sources

which are embryonic stem cells and adult stem cells, where the former comes from embryos and the latter originates from mature adults (Asahara *et al.*, 2000).

Embryonic stem cells (embryoblast) derive from a four- to six-day-old human embryo that is in the blastocyst phase of development (Figure 2.3). The embryos are usually extras that originated from *in vitro* fertilization (IVF) clinics. Sexual reproduction begins when a male's sperm fertilises a female's ovum (egg) to form a single cell called a zygote. The single zygote cell then begins a series of divisions, forming to 2 to 4, 4 to 8, 8 to 16, and so on. After 4 to 6 days post-fertilisation, a hollow ball of cell known as blastocyst is formed. The blastocyst consists of an inner cell mass (embryoblast) and an outer cell layer (trophoblast). The outer cell layer becomes part of the placenta, while the inner cell mass is the group of cells that will differentiate to become all the structures of an adult organism. This inner cell is known as embryonic stem cell which is a great therapeutic cell source to various health problems due to its potential to give rise to an entire organism and to differentiate into all cell lineages. However, the main concern with embryonic stem cells is the way that they are acquired which triggers ethical issue as it will results in the destruction of human embryo. Embryonic stem cells also divide uncontrollably, leading to growth of unwanted tissues and tumours called teratoma (Yamanaka, 2020).

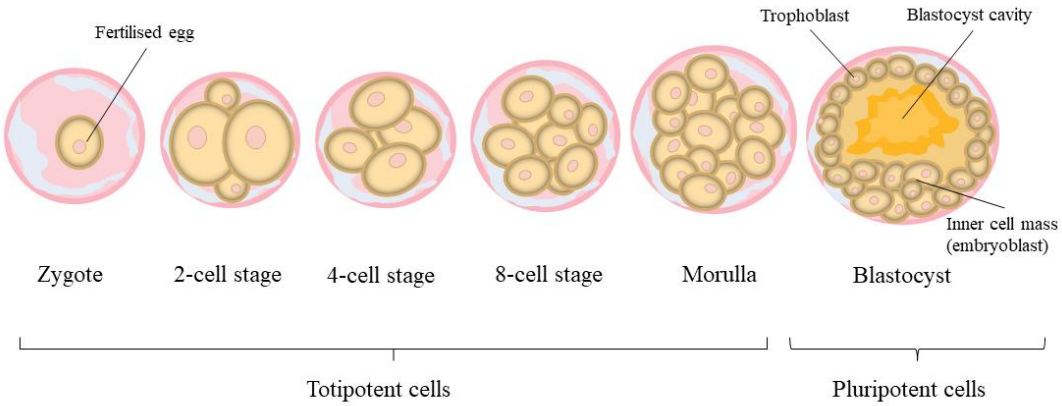

Figure 2.3 The development of zygote (fertilised egg) to blastocyst (inner cell mass). Adapted from (Łos *et al.*, 2018).

Adult stem cells are multipotent cells (can differentiate into many types of cells that originate from the same lineage) which are responsible for replacing damaged and dead cells in the body and can be divided into tissue-specific stem cells. These stem cells can be found in tissues such as the brain, bone marrow, blood, blood vessels, skeletal muscles, skin, and the liver. They remain in a quiescent or non-dividing state for years until activated by disease or tissue injury. Adult or somatic stem cells can divide or self-renew indefinitely, enabling them to generate a range of cell types from the originating organ or even regenerate the entire original organ (Carresi *et al.*, 2021). One of the tissue-specific adult stem cells are neural stem cells (NSC) which are located in the subventricular zone (SVZ) and subgranular zone (SGZ) or the dentate gyrus (DG) of the hippocampus in brain (Grochowski *et al.*, 2018).

2.2.2 Neural stem cell

NSCs are multipotent cells which generate progenitor cells that later primarily differentiate into neurons, astrocytes, and oligodendrocytes to maintain brain homeostasis (Łos *et al.*, 2018) (Figure 2.4). It was first isolated from the lateral

ventricle ependymal cell layer called the SVZ in the mouse brain by Reynold and Weiss in 1992 (Reynolds & Weiss, 1992). NSCs possess unique ability to mitigate stroke pathology since they are able to migrate through the CNS and repopulate lesion site after ischemic injury. There have been so many positive studies of ischemic stroke patients undergoing natural endogenous neurogenesis at the ischemic penumbra region of the brain (Guo *et al.*, 2022; Hamblin & Lee, 2021; Tornero, 2022). It was also reported that in the post-ischemic brain, dying neurons was replaced by new viable immature neuronal cells (neuroblasts) which were differentiated from endogenous NSCs originated from DG and SVZ (Kojima *et al.*, 2010). NSCs exhibit multiple potentially therapeutic actions against neurovascular inflammation (Hamblin & Lee, 2021). Besides, human NSCs are also known *in vivo* to promote new blood vessel growth (Sinden *et al.*, 2017).

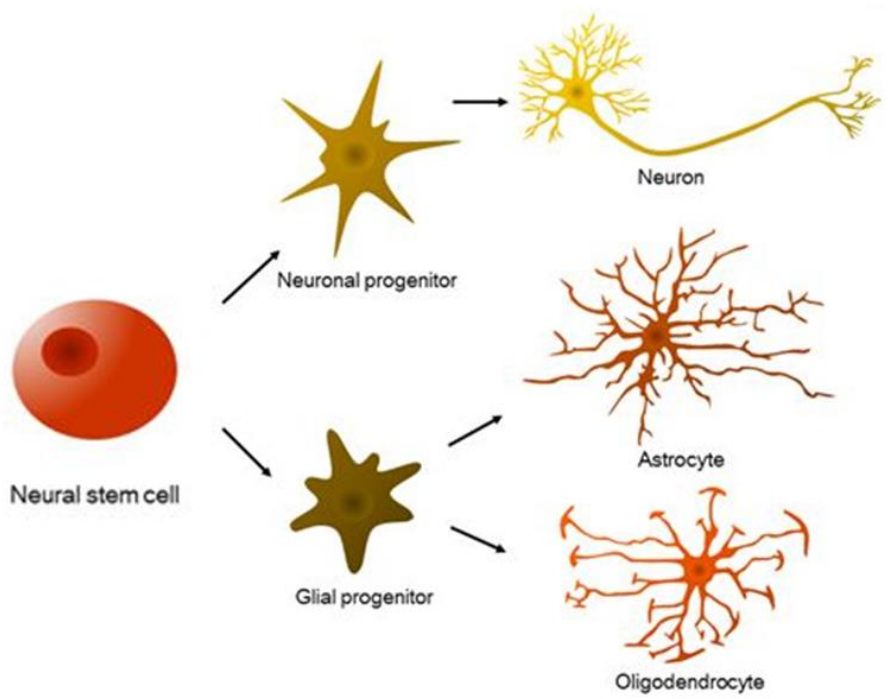

Figure 2.4 The derivatives of neural stem cells into neuron, astrocytes and oligodendrocytes. Adapted and edited from Tang (2017).

Besides endogenous NSC, exogenous NSC transplantation approach also is a good alternative for neurogenesis. Exogenous NSCs can be obtained from donor compatible with the recipient. Transplantation of NSCs interindividual or interspecies have lower immune rejection (Morizane *et al.*, 2017). Transplantation of NSCs into animal stroke model not only was found to restore the damaged brain tissue (neurogenesis), but also able to exert neuroprotection towards inflammation of brain tissue during the acute phase of the stroke. Besides, NSCs can easily cross the blood-brain barrier (BBB) and has the ability to migrate to the damaged site due to its strong chemotaxis and migration abilities (Addington *et al.*, 2017; Xu & Heilshorn, 2013). Another potentially attractive advantage of NSC therapy over conventional drug therapies is that NSCs can continually respond to environmental cues and secrete appropriate quantities and type of signalling factors, therefore providing a tailored response to individual stroke injuries (Baker *et al.*, 2019).

Nevertheless, the site of ischemic injury is usually associated with occluded blood flow, extracellular matrix degradation, oxidative stress, inflammation, and acute immune response. As a consequence, there are only limited transplanted stem cells or newly formed brain cells that can survive the hostile microenvironment in the ischemic regions, leaving only a few number of cells that will integrate to populate the target region for a prolonged duration in ischemic environment (Hicks *et al.*, 2009; Othman & Tan, 2020). Moreover, most of the endogenous NSCs also are rather dormant in normal setting to prevent their own exhaustion, lowering the retention rate in injured tissues thus limiting the cell proliferation and reducing the benefit of their therapeutic effect. Therefore, either endogenous or exogenous NSCs are capable to generate

sufficient viable new brain cells for structural or functional restoration of the injured brain.

In light of this, many researches have been conducted to prolong the NSCs survival in damaged tissues by minimising immune rejection and increase resistance against oxygen and nutrient deprivation, as well as oxidative stress in the ischemic area. Among those, stem cell preconditioning had generated much interest.

2.2.3 Preconditioning strategy

Stem cell preconditioning means exposing the cells to certain pharmacological, biological or physical inducers in order to improve cell resistance against the host harsh environment and to enhance their regulatory function of the local immune responses before transplantation into the recipient host (Moeinabadi-Bidgoli *et al.*, 2021). Preconditioning strategies have been tested in many types of stem cells, including NSCs, to increase cell survival rate and to maximize their therapeutic potential within ischemic microenvironments.

There are many approaches of preconditioning stem cells before cell grafting. For example, hypoxic preconditioning which involves brief periods of hypoxia that triggers various protective signalling pathways and enhances resilience to ischemia has been extensively investigated in various cell types, organs, animal models, and human. Zhuo (2021) reported that ischemic-hypoxic preconditioned olfactory mucosa mesenchymal stem cells protected mitochondrial function and inhibited apoptosis and pyroptosis of neurons in rat ischemia model. Other NSC preconditioning approaches

in treating ischemic injury are using cytokine such as interleukin-6 (Sakata, Narasimhan, *et al.*, 2012) or even antibiotics such as minocycline (Sakata, Niizuma, *et al.*, 2012).

Another promising approach is the brief period exposure of stem cells towards natural compound because of its more sustainable and environmentally friendly approach. Interestingly, pharmacological preconditioning represents a novel and efficient technique for stimulating the secretory activity of stem cells. Preconditioning with natural compound had successfully ameliorated ischemic nerve damage by enhancing the survival of transplanted stem cells under oxidative stress and increasing endogenous neural stem cell proliferation of ischemic nerve injury (Li *et al.*, 2020; Shu *et al.*, 2020). Therefore, in this study, NSC culture was preconditioned with brief periods of exposure to baicalein active compound extracted from *Oroxylum indicum* plant to enhance its bioactivity. *O. indicum* extract has been shown to exert neuroprotection effect and increase cell proliferation rate in many other cell types mainly due to its baicalein composition (Liu *et al.*, 2017; Van Leyen *et al.*, 2006; Zhang *et al.*, 2005).

2.3 Natural product for preconditioning as source of neuroprotective agent

2.3.1 Oroxylum indicum

Oroxylum indicum (L.) Vent., a semi-deciduous tree belongs to family *Bignoniaceae* have wide array of medicinal and ethnomedicinal properties (Jagetia, 2021). In Malaysia, this plant is locally known as beko, beka or bonglai. Other common names are “Midnight Horror” as the flowers bloom at night while discharging strong odour to attract their pollinators, “Indian trumpet tree” since the flower

resembles trumpet and “broken bones tree” due to accumulation of dry leaf and peduncle beneath the tree which resembles a pile of broken bones (Ahad *et al.*, 2012; Begum *et al.*, 2019; Kalra & Kaushik, 2017; Wahab *et al.*, 2018). The young shoots and unripe fruits of this plant are popularly consumed as vegetable or ‘ulam’ by the locals since it is claimed to have significant anti-aging properties and health benefits (Kang *et al.*, 2019; Salleh *et al.*, 2020; Singh, 2015). The plant is widely distributed throughout India, Nepal, Indonesia, Sri Lanka, Philippines, China, Japan, Bhutan, Malaysia, Taiwan, Thailand and Vietnam (Kalra & Kaushik, 2017).

O. indicum is a small to medium sized deciduous tree, of height 18-20 metres (Figure 2.5A). The flowers bloom on top of the tree and the fruits hang down from the bare branches like dangling swords (Figure 2.5B). Bark of the plant is light brown or greyish-brown coloured, which is soft and spongy with numerous corky lenticels. The leaves are pinnately compound which normally grows up to 3–10 cm long (Figure 2.5C) while the leafstalks of the adjacent leaflets are 6-15 mm long. The corolla of the flower is funnel shaped, about 10 cm long with 5 lobes, subequal with wrinkled margin, reddish outside, and yellowish to pinkish colored inside (Figure 2.5D). The flowers are bisexual, numerous in numbers and the flowers stalk is approximately 30 cm (Figure 2.5E). The fruits are woody, winged, large and flat, capsule or sword shaped (Figure 2.5F). The flowers usually bloom in rainy season and fruit appears in December to March (Jagetia, 2021; Kalra & Kaushik, 2017; Salleh *et al.*, 2020). This plant also lives in a mutualistic association with an actinomycete *Pseudonocardia oroxyli*, a gram-positive bacterium that has the capacity to produce many secondary metabolites exhibiting a wide variety of biological activities (Ahad *et al.*, 2012; Lalrinzuali *et al.*, 2018).