THE EFFECTS OF BAICALEIN EXTRACTED FROM *OROXYLUM INDICUM* LEAVES ON NEURAL STEM CELL GENE EXPRESSION

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

%	Percentage
ºC	Degree Celsius
µg	Microgram
µL	Microliter
ATP	Adenosine triphosphate
ddH_2O	Doubled distilled water
et al.	et alii – 'and others'
g	Gram
L	Litre
MCA	Middle cerebral artery
mL	Milliliter
mM	Milimolar
nm	Nanometer
nmol	Nanomole
NSCs	Neural stem cells
rpm	Revolutions per minute
TLC	Thin layer chromatography
tPA	tissue plasminogen activator
WSO	World Stroke Organization

KESAN BAICALEIN YANG DIEKSTRAK DARIPADA DAUN *OROXYLUM INDICUM* TERHADAP EKSPRESI GENE SEL STEM SARAF

ABSTRAK

Strok iskemia adalah disfungsi serebrum yang disebabkan oleh penyumbatan arteri di otak. Perubahan patofisiologi selepas strok iskemia mengakibatkan kematian sel, defisit neurologi, dan kecederaan otak. Walaupun beberapa rawatan strok iskemia kini tersedia, semua jenis rawatan berkenaan terhad oleh ketidakupayaan untuk menjana semula tisu otak yang rosak atau tidak berfungsi dalam otak iskemia. Oleh itu, terapi regeneratif menggunakan sel stem saraf boleh digunakan untuk rawatan strok iskemia kerana ia mempunyai keupayaan untuk membaiki, menggantikan dan menjana semula kawasan yang terjejas di dalam otak. Walau bagaimanapun, rawatan regeneratif menggunakan sel stem saraf mempunyai kadar kelangsungan sel yang rendah selepas dipindahkan ke otak iskemia akibat tekanan oksidatif dan tindak balas keradangan. Oleh itu, kajian ini mengaplikasikan strategi prasyarat menggunakan baicalein, sejenis flavonoid untuk menyiasat sama ada ia boleh meningkatkan sifat terapeutik sel stem saraf. Kajian ini bertujuan untuk menentukan kesan baicalein yang diekstrak daripada *Oroxylum indicum* terhadap potensi terapeutik sel stem saraf untuk strok iskemia dengan menjelaskan ekspresi gen. Penyelidikan ini menunjukkan bahawa Angiopoietin 1 (ANGPT1) ($p = 0.00001$) telah naik dengan ketara dengan perubahan lipatan tertinggi iaitu 5.9 diikuti Superoxide dismutase 2 (SOD2) ($p = 0.000003$) dengan perubahan lipatan 3.4 menunjukkan baicalein terfraksinasi boleh menggalakkan sifat angiogenesis dan sifat antioxidan dalam sel stem saraf untuk strok iskemia. Selain itu, Neuroepithelial stem cell protein (NESTIN) ($p = 0.039$) dan SRY-box transcription factor 2 (SOX2) ($p = 0.023$) telah naik dengan ketara dengan

perubahan lipatan 1.61 dan 1.25, menunjukkan bahawa ekstrak baicalein terfraksinasi boleh meningkatkan sifat stemness dalam sel stem saraf. Gamma-aminobutyric acid type A receptor subunit alpha 6 (GABRA6) (p=0.045) juga menunjukkan peningkatan yang ketara menerangkan bahawa baicalein berpotensi mengurangkan excitotoxicity dalam sel stem saraf yang disebabkan oleh strok iskemia. Walau bagaimanapun, keputusan yang tidak dijangka diperolehi dalam ekspresi Interleukin 1 receptor antagonist1 (IL-1RN). Gen ini tidak menunjukkan sebarang kesan ketara dalam sampel yang dirawat yang menunjukkan bahawa IL-1RN mungkin tidak menyumbang dalam mengurangkan keradangan dalam sel stem saraf. Kesimpulannya, kajian ini membentangkan membentangkan sifat baicalein yang boleh meningkatkan revaskularisasi, sifat antioksidan, pembaharuan diri, dan mengurangkan excitotoxicity dalam sel stem saraf.

THE EFFECTS OF BAICALEIN EXTRACTED FROM *OROXYLUM INDICUM* LEAVES ON NEURAL STEM CELL GENE EXPRESSION

ABSTRACT

Ischemic stroke is a cerebral dysfunction caused by the blockage of an artery in the brain. The pathophysiological events after ischemic stroke result in cell death, neurological deficits, and brain injury. Although several ischemic stroke treatments are currently available, they are limited by the ability to regenerate the damaged or dysfunctional brain tissue in the ischemic brain. Hence, regenerative therapy using neural stem cells (NSCs) can be applied to ischemic stroke treatment as it has the ability to repair, replace and regenerate the affected region in the brain. However, regenerative treatment using NSCs has a low cell survival rate after being transplanted into the ischemic brain due to oxidative stress and inflammatory response. Therefore, the present study utilized a preconditioning strategy using baicalein, a flavonoid to investigate whether it can enhance the therapeutic properties of NSCs. This study aimed to determine the effects of baicalein extracted from *Oroxylum indicum* on the therapeutic potential of NSCs for ischemic stroke by elucidating the gene expression. The findings showed that Angiopoietin 1 (ANGPT1) ($p = 0.00001$) was significantly upregulated with the highest fold change which is 5.9 followed by Superoxide dismutase 2 (SOD2) ($p = 0.000003$) with fold change 3.4 indicating that fractionated baicalein can promote angiogenesis and antioxidant properties in NSCs for ischemic stroke. Besides, Neuroepithelial stem cell protein (NESTIN) ($p = 0.039$) and SRY-box transcription factor 2 (SOX2) ($p = 0.023$) were significantly upregulated with fold change 1.61 and 1.25 respectively, indicating that fractionated baicalein extract could improve the stemness property of NSCs. Gamma-aminobutyric acid type A receptor

subunit alpha 6 (GABRA6) (p=0.045) also showed significant upregulation suggesting that baicalein can potentially reduce excitotoxicity in NSCs caused by ischemic stroke. However, unexpected result was obtained in the expression of Interleukin 1 receptor antagonist (IL-1RN). This gene did not show any significant effect in the treated samples which demonstrates that IL-1RN might not contribute in reducing inflammation in NSCs. In conclusion, this study presented that baicalein active compound could enhance revascularization, antioxidant properties, self-renewal, and reduce excitotoxicity in NSCs.

CHAPTER 1

INTRODUCTION

1.1 Introduction

A stroke is defined as rapidly developed clinical signs of cerebral dysfunction, which lasts more than a day or triggers death with no apparent cause other than vascular origin (Alrabghi et al., 2018). Ischemic stroke and hemorrhagic stroke are the two major types of strokes. Ischemic stroke is caused by the blockage of an artery in the brain whereas hemorrhagic stroke is caused by the rupture of a blood vessel in the brain (Barthels & Das, 2020). Both of these strokes cause local hypoxia that eventually leads to brain tissue injury. However, several studies reported that ischemic stroke is more prevalent compared to hemorrhagic stroke (Feigin et al., 2022; Rennert et al., 2019). The causes of ischemic stroke are thrombosis, hypo-perfusion, and embolism which decrease the blood flow to the brain (Alrabghi et al., 2018). The risk factors of ischemic stroke are hypertension, diabetes mellitus, increased cholesterol, and lipid levels, smoking, sedentary lifestyle, obesity, and family history (Rennert et al., 2019).

During ischemic stroke, an artery in the brain is blocked, causing reduced blood flow to the brain. The lack of glucose and oxygen leads to reduced levels of adenosine triphosphate (ATP) in the brain resulting in the malfunction of calcium-potassium pumps and neuronal depolarization. This causes excitotoxicity due to the continuous stimulation of glutamate. Consequently, increased production of nitric oxide and reactive oxygen species results in oxidative stress which eventually leads to mitochondrial, metabolic failure, and DNA damage. As a result, neuroinflammation occurs in the ischemic brain and leads to further tissue damage and accumulations of

cytokines to toxic levels. These pathophysiological changes, in the end, affect the brain tissue, blood vessels, and blood-brain barrier resulting in loss of brain integrity (Majid & Kassab, 2022).

Currently, there are several treatments available for ischemic stroke which include thrombolytic therapy in which the patient will be given a drug known as tissue plasminogen activator (tPA) that dissolves the clot that is blocking the blood flow to the brain, and thrombectomy, the procedure of physically removing the clot and widening the blood vessel (Hui et al., 2022; Barthels & Das, 2020; Herpich & Rincon, 2020). Besides, antithrombotic treatment such as antiplatelet drugs, anticoagulants including drugs to reduce blood pressure and cholesterol, and stroke rehabilitation are important therapies in ischemic stroke management (Hui et al., 2022; Barthels & Das, 2020; Alrabghi et al., 2018). Nevertheless, these current treatments are unable to regenerate the damaged ischemic region in the brain. To date, there are no clinically effective treatments for the damage caused by an ischemic stroke (Hurd et al., 2021).

Thus, regenerative treatment using neural stem cells (NSCs) has great potential to be an effective treatment for ischemic stroke because it can repair and replace damaged tissues in the ischemic region. This is supported by the studies that reported successful expansion, differentiation, neuronal regeneration, and angiogenesis in experiments using the transplantation of stem cells (Barthels & Das, 2020).

NSCs are undifferentiated multipotent stem cells that can self-renew, proliferate, and differentiate into neurons and glial cells that give rise to the central nervous system during development (Galiakberova & Dashinimaev, 2020; Tabansky & Stern, 2016).

NSCs hold great potential in treatments for cerebrovascular and neurodegenerative diseases. However, NSCs have a very low survival rate in vivo because the microenvironment in the ischemic area undergoes oxidative stress, neuroinflammation, and immune response which reduces the ability of the stem cells to replace the damaged cells (Li et al., 2022)

This limitation can be overcome by preconditioning the NSCs ex vivo to enhance the ability of the NSCs after transplantation. In this study, a natural product known as baicalein was used to precondition the NSCs. Baicalein is a natural flavonoid that is rich in many pharmacological properties. Baicalein possesses neuroprotective properties as it reduces oxidative stress, and neuroinflammation and inhibits the formation of clots in blood vessels (Li et al., 2022; Jugait et al., 2022; Hui et al., 2022). In this study, baicalein was extracted from the leaves of *Oroxylum indicum*, a plant commonly known as Beko by local Malaysian (Rojsanga et al., 2020).

1.2 Problem Statement

Current treatments for ischemic stroke primarily focus on removing the clot that is blocking the blood flow to the brain, preventing secondary stroke attack and rehabilitation. Currently, there is no treatment that can generate the damaged cells in the brain caused by ischemic stroke. Regenerative therapy using NSCs offers an alternative treatment to repair, replace or regenerate damaged or dysfunctional neural tissues. However, a low cell survival rate after transplantation limits NSC-based regenerative therapy. Thus, this study utilizes neuroprotective baicalein active compound with anti-oxidant and anti-inflammatory properties to enhance the regenerative therapy using NSCs.

1.3 Significance of study

Ischemic stroke poses a significant burden on patients and healthcare systems. By investigating the therapeutic potential of baicalein-preconditioned NSCs, this study seeks to contribute crucial insights into regenerative medicine for ischemic stroke. The findings of this study may provide a basis for developing effective treatment strategies to repair and regenerate damaged tissues and reverse the pathophysiological changes caused by ischemic stroke. The study outcomes can also have great implications for NSC research, particularly in NSC-preconditioning strategy utilizing natural products.

1.4 Objectives

1.4.1 General Objective

To determine the effects of baicalein extracted from *O. indicum* on the therapeutic potential of NSCs for ischemic stroke by elucidating the gene expression.

1.4.2 Specific Objectives

1. To extract and enrich baicalein from *O. indicum* leaves.
2. To investigate the effect of baicalein extracted from *O. indicum* on the anti-oxidant property of NSCs.
3. To investigate the effect of baicalein extracted from *O. indicum* on the anti-inflammatory property of NSCs.
4. To investigate the effect of baicalein extracted from *O. indicum* on the neuroprotective property of NSCs.

1.5 Hypothesis

NSCs treated with fractionated baicalein extract will significantly enhance the antioxidant, anti-inflammatory, and neuroprotective properties of NSCs.

1.6 Study framework

The study framework of this research project is shown in Figure 1.1.

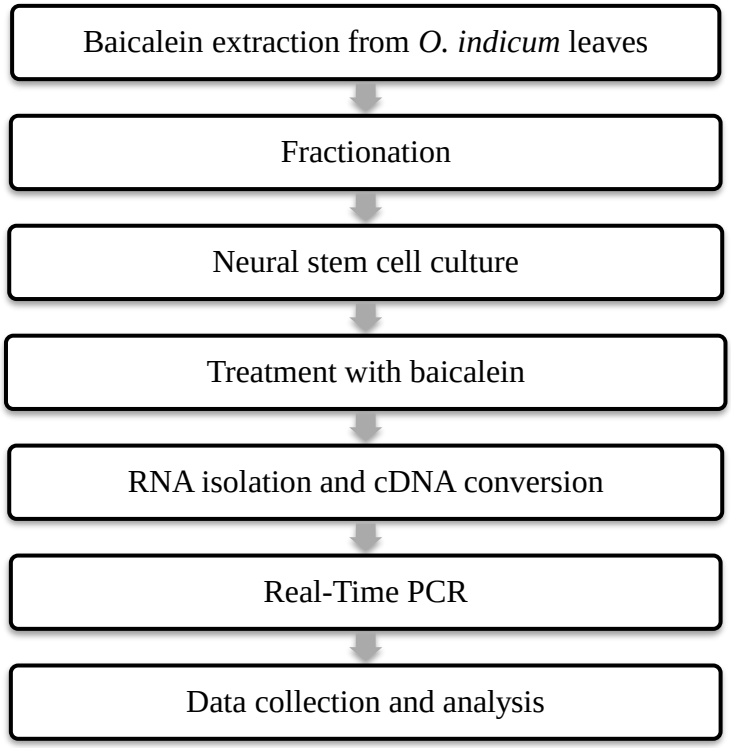

Figure 1.1: The study framework

CHAPTER 2

LITERATURE REVIEW

2.1 Ischemic stroke

The World Health Organization (WHO) defines stroke as a sudden onset of clinical symptoms of brain dysfunction that last for more than a day or result in death but have no other known cause than vascular origin (Alrabghi et al., 2018). Stroke is the third leading cause of mortality, causing severe morbidity and negative impacts on society in Malaysia (Tan & Venketasubramanian, 2022). It is indicated that the incidence rate of ischemic stroke will increase in many countries between the years 2020 and 2030 (Pu et al., 2023).

An ischemic stroke occurs when a blood clot or other blockage reduces or completely blocks blood flow to a part of the brain whereas a hemorrhagic stroke occurs when a blood vessel in the brain ruptures or leaks, causing bleeding into or around the brain tissue (Barthels & Das, 2020). Both of these strokes eventually result in brain tissue injury due to hypoxia. The Global Stroke Fact Sheet 2022 by The World Stroke Organization (WSO) reported the incidence of new ischemic stroke each year is more than 7.6 million and almost 11% of the ischemic strokes occur in the young population aged 15 to 49 years old (Feigin et al., 2022).

2.1.1 Risk factors of ischemic stroke

Several clinical and behavioral conditions contribute to a higher risk of having an ischemic stroke. The risk factors for stroke are either modifiable such as behavioral changes or non-modifiable which includes a positive family history of strokes, age,

and male gender (Alrabghi et al., 2018). Besides, hypertension, diabetes mellitus, increased cholesterol, unhealthy lifestyle, and smoking are also the risk factors of ischemic stroke (Rennert et al., 2019). Other risk factors that lead to ischemic stroke include arteriosclerosis, vasculitis, arterial dissection, and small vessel disease (Hui et al., 2022).

2.1.2 Causes of ischemic stroke

The main etiologies of ischemic stroke are thrombosis and embolism (Alragbhi et al., 2018). These events cause a decrease in blood flow to the brain leading to ischemic stroke. In a thrombotic event, the blood flow to the brain is blocked by a blood clot, known as a thrombus that forms and remains in the blood vessel; while in an embolic event, an embolus which is originated from another location in the body blocks the blood flow to the brain (Hui et al., 2022). When an artery in the brain is blocked, the blood flow to the brain parenchyma becomes inadequate, and this results in cellular bioenergetic failure and an inflammatory cascade that consequently leads to the death of neurons, glia, and endothelial cells in the ischemic area (Rennert et al., 2019).

2.1.3 Clinical symptoms of ischemic stroke

Ischemic stroke symptoms depend on the exact part of the brain affected, which has decreased blood flow. The specific area of the brain affected by stoke plays a significant role in determining the clinical symptoms as different parts of the brain control various functions. For example, an ischemic stroke at the middle cerebral artery (MCA) can cause dysarthria which is speaking difficulty due to muscle weakness whereas an ischemic stroke at the posterior cerebral artery can affect vision (Hui et al., 2022). Besides, ischemic stroke progression and severity mainly depends on the

volume of infarcted tissue which is determined by the degree and length of hypoperfusion and the level of collateral flow to an ischemic region (Rennert et al., 2019). Larger infarcts may result in more severe deficits and potentially life-threatening complications.

2.1.4 Pathophysiological changes after ischemic stroke onset

There are several pathophysiological changes that take place after an ischemic stroke as shown in Figure 2.1, which eventually leads to brain injury (Majid & Kassab, 2022; Qin et al., 2022; Mariman et al., 2022). During ischemic stroke, there is a reduction of blood flow to the brain as the blood vessel is blocked. Reduced blood flow leads to decreased levels of adenosine triphosphate (ATP) in the brain due to the lack of glucose and oxygen. As a result, energy deficiency causes ionic imbalance which leads to malfunction of calcium-potassium ion pumps leading to neuronal depolarization. The immediate deprivation of glucose and oxygen causes neurons to lose their ability to produce electrochemical gradient. Consequently, excitotoxicity occurs due to the efflux of potassium and the influx of sodium and calcium ions besides the continuous stimulation of glutamate. The excessive glutamate induces toxicity, cell death and severe damage to the central nervous system. Excitotoxicity leads to oxidative stress due to the increased production of nitric oxide and reactive oxygen species. Oxidative stress results in several consequences such as mitochondrial and metabolic failure including DNA damage. Besides, ischemic stroke also activates the inflammatory pathway in the ischemic region. Although inflammation aids in removing damaged cells in the ischemic region, it can also lead to tissue damage and accumulations of cytokines to toxic levels. Eventually, all these consequences lead to loss of brain

integrity as the brain tissue, neuronal networks, blood vessels, and blood-brain barrier are severely affected due to ischemic stroke.

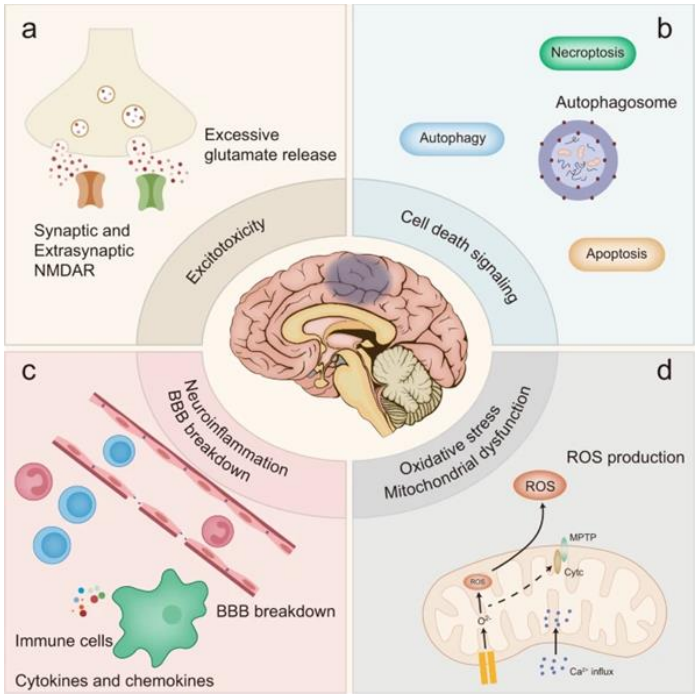

Figure 2.1: Summary of pathophysiological effects caused by ischemic stroke

(Source: Qin et al., 2022)

2.1.5 Current treatments for ischemic stroke

When an ischemic stroke happens, the blood supply to part of the brain is interrupted, depriving the brain tissues of oxygen and nutrients resulting in brain cell death. Thus, immediate treatment is crucial to promote revascularization and to reduce the chances of secondary neuronal injury as well as unwanted complications.

Currently, the available treatments for ischemic stroke include thrombolytic therapy. It is one of the early treatments for ischemic stroke which involves giving a thrombolytic drug known as tissue plasminogen activator (tPA) that dissolves the clot that is blocking the blood flow to the brain through the activation of plasminogen to

plasmin (Barthels & Das, 2020; Herpich & Rincon, 2020). Alteplase and Tenecteplase are examples of thrombolytic drugs used for ischemic stroke treatment (Herpich & Rincon, 2020). Patients are likelier to have minimal or no disability if tPA is administered within the first three hours of symptoms (Herpich & Rincon, 2020). However, treatment with tPA outside the therapeutic time window can lead to hemorrhagic transformation and further brain damage (Barthels & Das, 2020). Besides, the pharmacological management of ischemic stroke also includes antithrombotic treatment such as antiplatelet drugs and anticoagulants and drugs to reduce blood pressure and cholesterol in order to prevent secondary stroke attack (Barthels & Das, 2020; Hui et al., 2022).

On the other hand, thrombectomy is the procedure of physically removing the clot and widening the blood vessel when treatment with tPA or other drug is not applicable to the patient or when the patient arrives at the hospital later than expected (Barthels & Das, 2020; Herpich & Rincon, 2020; Hui et al., 2022). Stroke rehabilitation is also another therapy used to help patients during the recovery phase to relearn movements and coordination skills that they lost due to a stroke (Alrabghi et al., 2018). However, these treatments share a common limitation, which they are unable to regenerate the damaged ischemic region in the brain.

2.1.6 Emerging research and strategies for the treatment of ischemic stroke

The currently available treatments for ischemic strokes are extremely limited to treat and lessen the disabilities that ischemic stroke can cause. There are no clinically effective treatments for the damage caused by an ischemic stroke up to date (Hurd et al., 2021).

Apart from these currently practiced treatments, there is much ischemic stroke research being conducted to develop advanced therapies for better clinical outcomes. Thus, a lot of studies are being conducted in this area. For instance, the use of regenerative therapies which have the potential to heal and replace damaged tissues. Successful expansion, differentiation, neuronal regeneration, and angiogenesis in experiments using transplantation of stem cells show that stem cells have great therapeutic potential in ischemic stroke treatment (Barthels & Das, 2020).

2.2 Neural Stem Cells (NSCs)

2.2.1 Characteristics of NSCs

Stem cells are defined as cells that can divide to self-renew and differentiate into other cell types depending on their potency (Singh et al., 2016; Zhao & Moore, 2018). Stem cells in general are categorized into unipotent, multipotent, and pluripotent (Singh et al., 2016). The important feature of stem cells is to produce specialized cells to perform specific functions in the body system (Swayne et al., 2016). NSCs are undifferentiated multipotent stem cells that produce multiple cells within a particular lineage, meaning they self-renew and proliferate to produce cells that differentiate into neurons and glial cells that give rise to the central nervous system during development (Galiakberova & Dashinimaev, 2020b; Tabansky & Stern, 2016). Neurons transmit and receive signals while glial cells, which include oligodendrocytes, astrocytes, and microglial cells, support the neurons by enhancing signal transmission speed (Swayne et al., 2016).

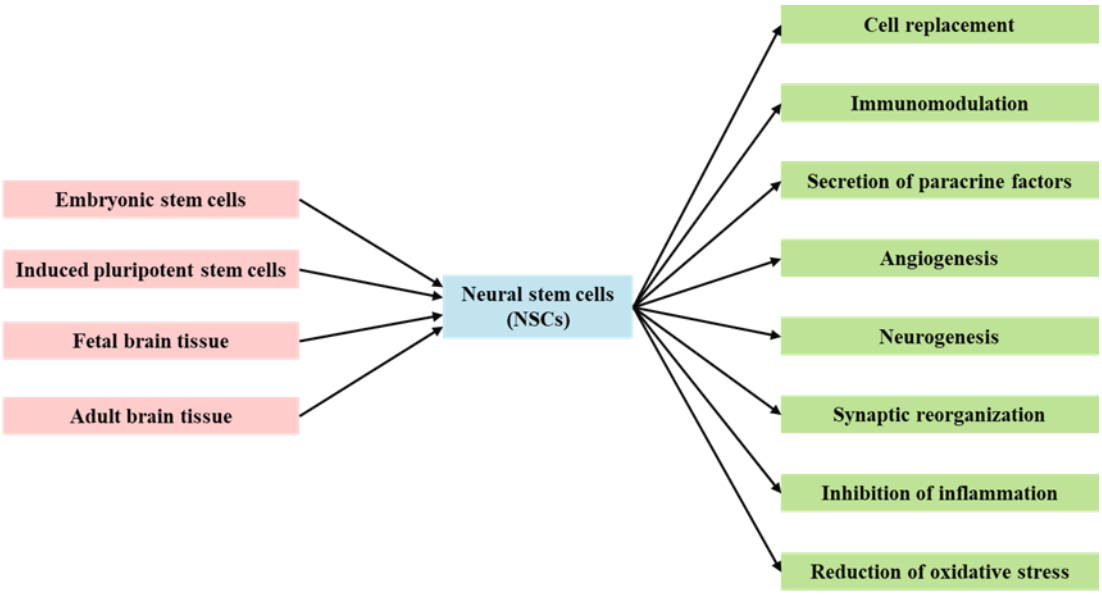

Figure 2.2: Sources and therapeutic potentials of NSCs

NSCs can be derived from embryonic stem cells, induced pluripotent stem cells, and fetal and adult brain tissue as shown in Figure 2.2. In the fetal stage, NSCs can be isolated from the developing cerebral cortex, midbrain, and retina whereas in the adult brain, NSCs are found specifically in the subventricular zone of the lateral ventricle and the subgranular of the hippocampal dentate gyrus.

2.2.2 Potential of NSC therapy for ischemic stroke

There are some key aspects of NSC potential in ischemic stroke as illustrated in Figure 2.2. Pathophysiology of ischemic stroke involves oxidative stress, increased apoptosis, neuroinflammation, neuronal injury, and brain tissue damage. NSC transplantation have the potential to reverse these detrimental effects. NSC therapy can promote cell migration and differentiation to replace damaged brain tissue and inhibit oxidative stress, apoptosis, and inflammation in the ischemic region (Kawabori et al., 2020; Liu et al., 2021). Besides, NSCs also secrete various paracrine factors such as cytokines and chemokines, which can ameliorate neuronal damage by ischemic stroke(Kawabori

et al., 2020). Furthermore, NSCs exhibit immunomodulatory function, promote angiogenesis and enhance neural repair mechanisms which include neurogenesis and neuronal reorganization (Baker et al., 2019).

2.2.3 Challenges in utilizing NSCs in clinical trials

NSCs are being studied extensively to be used as treatments for diseases and injuries related to the nervous system as they hold great potential to replace lost neurons, strengthen damaged synaptic networks and repair injured brain (Yue et al., 2022). For instance, researchers continue to assess the therapeutic potential of NSCs in Alzheimer's disease, Parkinson's disease, Huntington's disease, and spinal cord injury including stroke (Yue et al., 2022; Zhao & Moore, 2018). However, the challenge in utilizing NSCs is the grafting efficiency or survival rate of stem cells is lower than five percent in vivo (Zhang et al., 2019). Oxidative stress, neuroinflammation, and immune responses in the ischemic region are among the culprit to affect the survival of cells after the transplant, thereby reducing the ability of the stem cells to replace the damaged cells in the ischemic area (Zhang et al., 2019). Therefore, alternative approaches must be found to overcome these obstacles so that NSCs could be utilized to the maximum in clinical trials.

2.3 Preconditioning strategy in stem cell technology

Preconditioning refers to the strategy of using a variety of techniques to enhance the ability or potential of substances in ex vivo (Zhao et al., 2020). As mentioned earlier, NSCs are unable to survive and regenerate in the hostile environment of the ischemic region due to a lack of glucose and oxygen. Thus, a preconditioning strategy is needed to optimize the therapeutic potential of NSCs in the ischemic region. Preconditioning

stem cells prior to transplantation increases cell survival, proliferation, and angiogenesis through various molecular mechanisms and pathways (Moeinabadi-Bidgoli et al., 2021).

There are several examples of preconditioning approaches such as preconditioning with biological molecules and mediators, physical stimulants, acidosis, and serum deprivation (Moeinabadi-Bidgoli et al., 2021). Another important preconditioning strategy intensively being studied at the current moment is the hypoxic-preconditioned strategy (He et al., 2021; Liu et al., 2021; Moeinabadi-Bidgoli et al., 2021).

2.3.1 Hypoxic-preconditioned strategy

Hypoxic preconditioning refers to the strategy of giving the body a short period of mild hypoxic stimulus which can greatly improve the body's tolerance to more severe hypoxia events (Liu et al., 2021). In other words, hypoxic-preconditioned NSCs are expected to survive and regenerate in the ischemic area of the brain. Hypoxia Inducible Factor 1 Subunit Alpha (HIF-1α) is the master transcription factor in hypoxic stress adaptation. In the hypoxic-preconditioning strategy, HIF-1α can be upregulated by decreasing the concentration of oxygen or adding hypoxia mimetic agents such as cobalt to the cell culture milieu (Moeinabadi-Bidgoli et al., 2021). Besides, studies have shown that hypoxia increases differentiation, upregulates stemness genes such as SRY-box transcription factor 2 (SOX2), and activates angiogenic genes which include vascular endothelial factor (VEGF) (He et al., 2021). However, the main challenge is the benefit of hypoxic preconditioning last for a very limited period of time (Liu et al., 2021). This makes the laboratory work more challenging and tedious. Nevertheless,

HIF-1α can be induced in stem cells through natural products without hypoxic preconditioning which is more practical, feasible, and cost-effective.

2.4 Natural product-preconditioning strategy

A natural product refers to a chemical compound made by a living organism. Natural products play an important role in drug discovery and therapeutic fields. In general, plant-derived natural products have therapeutic potential in treating health conditions. For instance, totarol, taraxasterol, britanin, emodin, and ferulic acid are examples of natural compounds with oxidative stress-reducing potential in ischemic stroke (Tao et al., 2020). Baicalein is also a natural product that possesses various pharmacological and neuroprotective properties and it has been reported that baicalein can also activate HIF-1α (Wang et al., 2020). Therefore, baicalein is a potential therapeutic candidate for ischemic stroke treatment.

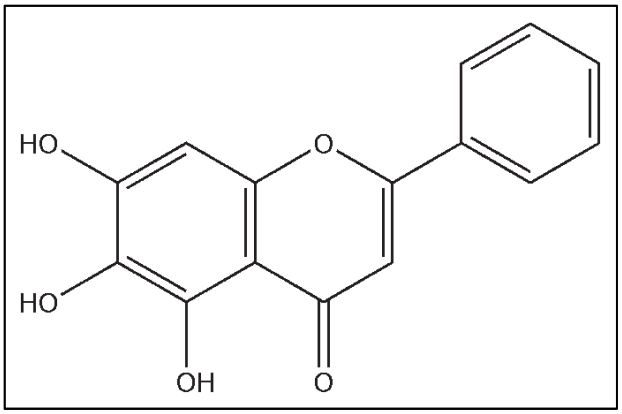

Figure 2.3: Chemical structure of baicalein compound

Baicalein, also known as 5,6,7-trihydroxyflavone (Figure 2.3), is a natural flavonoid that has many pharmacological activities such as anti-bacterial, anti-viral, anti-tumor, anti-inflammatory, and anti-thrombotic properties (Li et al., 2022; Jugait et al., 2022;

Zhao et al., 2022). Baicalein can be an effective therapeutic agent for ischemic stroke because it could penetrate the blood-brain barrier and reach the cerebral nuclei (Li et al., 2022).

Baicalein reduces oxidative stress as studies have reported that baicalein can alleviate the injury caused by cerebral ischemia-reperfusion through inhibition of ferroptosis and attenuate oxaliplatin-induced reactive oxygen species and mitochondrial superoxide levels (Jugait et al., 2022; Li et al., 2022). Besides, baicalein also reduces neuroinflammation by decreasing proinflammatory markers and elevating anti-inflammatory markers *in vivo* and *in vitro* (Ren et al., 2021). In addition, baicalein ameliorates the infarct volume caused by ischemic stroke (Li et al., 2022; Pan et al., 2021).

2.4.2 *Oroxylum indicum* medicinal plant as a source of baicalein

Baicalein can be found abundantly in a local medicinal plant known as *Oroxylum indicum*. *O. indicum* is a plant that belongs to the family Bignoniaceae, and it is commonly known as Beko by local population (Abdulhafiz et al., 2022; Rojsanga et al., 2020). The different parts of the plant such as fruit, seeds, leaves, stems, barks, and roots are used in folk and ayurvedic medicinal practices for many health which include asthma, fever, bronchitis, diarrhea, inflammation, wound injury, and arthritis (Jagetia, 2021).

O. indicum contains a variety of phytochemicals such as tannins, alkaloids, saponin, flavonoids, phenols, and oils and the most abundant active compound found in this plant is baicalein (Adin et al., 2022). Since *O. indicum* is widely distributed throughout

Asian countries, including Malaysia, it is easily found and consumed by locals as an important culinary herb (Abdulhafiz et al., 2022).

2.5 Natural product extraction and purification

The isolation of natural compounds from natural sources begins with an extraction process that provides the crude extract. Solvent extraction is one of the common extraction techniques. In general, solvent extraction comprises of few stages which include the solvent penetrating into the solid matrix, the solute dissolving in the solvent, then the solute diffusing out of the solid matrix and finally collecting the extracted solutes (Zhang et al., 2018). An example of solvent extraction technique is the Soxhlet extraction. Selecting an appropriate solvent is crucial to obtain the target compounds. For instance, Soxhlet extraction using petroleum ether followed by methanol produced the highest baicalein content compared to methanol alone and distilled water(Kang et al., 2019). The extraction is followed by a separation process that allows to obtain a purer desired natural compound. For instance, fractionation using column chromatographic technique is a method used to isolate a pure compound from the crude extract obtained in the extraction procedure (Abubakar & Haque, 2020).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 List of chemicals and reagents

Chemicals and reagents used in this study are listed in Table 3.1.

Table 3.1 List of chemicals

Chemical / Reagent	Manufacturer
Baicalein synthetic compound	Gibco, USA
Basic Fibroblast Growth Factor (bFGF)	Gibco, USA
Chloroform	Merck, Germany
CELLStart™ Defined, Humanized Substrate for cell culture	Systerm, US
Dulbecco's Phosphate Buffered Saline (D-PBS), containing no calcium, magnesium, or phenol red	Gibco, USA
Dulbecco's Phosphate Buffered Saline (D-PBS), containing calcium and magnesium, but no phenol red	Gibco, USA
Ethanol	R&M Chemicals, India
Epidermal Growth Factor (EGF)	HMBG, Germany
GlutaMax-1 supplement	Gibco, USA
Ion exchange resin (Diaion HP20)	Mitsubishi Chemicals (Japan)
Methanol	HMBG, Germany
Petroleum ether	Bendosen, Malaysi
Penicillin-Streptomycin	Gibco, USA
qPCRBIO SyGreen Mix Separate-ROX	PCR Biosystems, UK
RNeasy® mini kit	Qiagen, Germany
RNase free water	Gibco, USA
Serum free media Knockout DMEM/F-12	Gibco, USA
StemPro™ NSC SFM Supplement	Gibco, USA

Table 3.1 Continued

Chemical / Reagent	Manufacturer
Strand cDNA Synthesis Kit	Qiagen, Germany
Trypan blue Stain	-

3.1.2 List of consumables

Consumables used in this study are listed in Table 3.2.

Table 3.2 List of consumables

Consumable	Manufacturer
Cell counting chamber slides	Invitrogen, USA
Cell culture vessels (T25 flask, 24 wells, culture dish)	SPL Lifesciences, Korea
Centrifuge tubes (15 mL and 50 mL)	Biologix, USA
Glassware	DURAN, Germany
Microcentrifuge tubes (1.5 mL and 2 mL)	Axygen, USA, Germany
PCR tube	Axygen, USA
Pipette tips	Axygen, USA
Plastic paraffin film	Heathrow Scientific, USA
TLC plate	Merck, Germany
Whatman^{TM} Cellulose Soxhlet extraction thimble	Cytiva, USA

3.1.3 List of equipment

Equipment used in this study is listed in Table 3.3.

Table 3.3 List of equipment

Equipment	Manufacturer
-20 °C freezer	Haier, China
-80 °C deep freezer	ilShin, Korea
Analytical balance	A&D Company, Japan
Centrifuge mikro 22R	Hettich, Germany
Centrifuge universal 320	Hettich, Germany
CFX Opus 96 Real-Time PCR System	Bio-Rad, USA
CO_2 incubator	ESCO Lifesciences, Singapore
Countess™ automated cell counter	Thermo Fisher Scientific, USA
CytoSMART live-imaging system	Axion Biosystems, USA
Electronic balance	Shimadzu, Japan
Inverted microscope	Leica, Germany
Medicine blender powder grinder	-
Microbiological incubator	Binder, Germany
Nanodrop Spectrophotometer	Thermo Fisher Scientific, USA
Pipette (10 µL, 100 µL and 1000 µL)	Thermo Fisher Scientific, USA
Refrigerator	Samsung, Malaysia
Rotary evaporator	Heidolph, Germany
Soxhlet apparatus	PLT Scientific, Malaysia
Ultraviolet lamp and viewing cabinet	-
Veriti™ 96-well thermal cycler	Life Technologies, Singapore

3.1.4 List of cell line

In this study, the rat fetal NSCs (N7744-100) was purchased from Thermo Fisher Scientific, USA. These rat fetal NSCs were isolated from the cortexes of the fetal (embryonic day 14) Sprague-Dawley™ rats.

3.1.5 List of primers

Primers used in this study are listed in Table 3.4.

Table 3.4 List of primers

Gene name	Gene symbol	Function	Forward (5' to 3') Reverse (5' to 3')
SRY-box transcription factor 2	SOX2	Maintains the self-renewal and multipotent differentiation of neural stem cells	CAC ATG AAG GAG CAAC CCG GA CCT CCG GGA AGC GTG TAC TT
Neuroepithelial stem cell protein	NESTIN	Regulates the assembly and disassembly of Intermediate filament during mitosis	TGG AGC AGG AGA AGC AAG GT CCT CCA GCA GAG TCC TGT ATG T
Superoxide dismutase 2	SOD2	Antioxidant protein that protects cells against mitochondrial superoxide	TGG CGG GGG CCA TAT CAA TC CTC CAG CAA CTC TCC TTT GGG T
Interleukin 1 receptor antagonist	IL-1RN	Exerts anti-inflammatory activity	GGA AAT CTG CAG GGG ACC TTA TGG TTA GTA TCC CAG ATT CTG AAG G
Angiopoietin 1	ANGPT1	Regulates blood vessels and maintains stem cells	TCA GTG GCT GCA AAA ACT TGA GA ATG GTG GCC GTG TGG TTT TG

Table 3.4 Continued

Gene name	Gene symbol	Function	Forward (5' to 3') Reverse (5' to 3')
Gamma-aminobutyric acid type A receptor subunit alpha 6	GABRA6	Major inhibitory neurotransmitter in brain	TTG ACA ACT TGC TGG AGG GC ACA TCC ATT GTG TAC TCC ATC TCC A
Hypoxanthine phosphoribosyltransferase 1	HPRT1	Housekeeping gene: regulates multiple developmental and metabolic pathways in neuronal differentiation	AGT CCC AGC GTC GTG ATT AGT CGA GCA AGT CTT TCA GTC CTG TC
Ribosomal protein L13A	RPL13A	Housekeeping gene: Protein biosynthesis	GCT GCC GCA CAA GAC CAA AA CCA CCA TCC GCT TTT TCT TGT CA

3.2 Preparation

3.2.1 NSC culture

3.2.1.1 Complete media for rat fetal NSCs

In order to culture rat fetal NSCs, complete media was prepared using 48.5 mL of KnowOut DMEM/ F-12, 0.5 mL of 1.0% GlutaMAX-1 supplement, 0.01 mL of 0.02% Basic Fibroblast Growth Factor (bFGF), 0.01 mL of 0.02% Epidermal Growth Factor (EGF) and 1 mL of 2.0% StemPro neural supplement.

3.2.1.2 Coating of cell culture flask

30µL CellStart^{TM} Defined, Humanized Substrate was diluted in 3 mL D-PBS containing calcium and magnesium. 3 mL of the working solution was dispensed into T25 flask and then wrapped with aluminium foil. The flask was incubated in a humidified incubator at 37 °C for 1 hour. Then the working solution was discarded from the flask and 2 mL of D-PBS without calcium and magnesium was added into the flask. The flask was wrapped with aluminium foil and incubated in a humidified incubator with 5% CO_2 at 37 °C until further use.

3.2.1.3 Reviving rat fetal NSCs

The vial containing rat NSCs was thawed from cryopreservation storage in a water bath pre-set at 37 °C. The thawed cells were transferred into 15 mL tube. 4 mL of complete media was added drop-wise manner while swirling the tube. Then another 4 mL of complete media was added into the tube. The tube was centrifuged at 1500 rpm for 4 minutes at room temperature. The supernatant was discarded and 1 mL of complete media was added to re-suspend the cell pellet. Next, cell counting was done by mixing 10 µL of cell suspension with 10 µL Trypan blue. 10 µL of the mixture was

pipetted onto automated cell counting chamber slides and placed into an automated cell counter. The D-PBS in the pre-coated flask was pipetted out and 4 mL of fresh complete media was added into the flask. The cell suspension was transferred into T25 flask at a cell density of 1 x 10^5 cells/ flask. The flask was placed in humidified incubator with 5% CO_2 at 37 °C for 24 hours. The old media was then replaced with 4 mL of fresh complete media.

3.2.1.4 Passaging of rat fetal NSCs

The NSCs need to be passaged when they reach between 80% to 90% confluent to avoid cell death. The used media in the flask was discarded. 2 mL of D-PBS without calcium and magnesium was added into the flask to rinse the surface of cell layer. The D-PBS was discarded and 1 mL of Trypsin was added into the flask. The flask was incubated for 2 to 5 minutes at room temperature. Then, the flask was gently tapped and observed under light microscope to ensure complete cell detachment. 2 mL of complete media was added into the flask, and the detached cells were transferred into 15 mL tube and centrifuged for 5 minutes at 1500 rpm at room temperature. The supernatant was discarded and the cell pellet was re-suspended with complete media. Cell counting was done. The D-PBS in the pre-coated flask was pipetted out and 4 mL of fresh complete media was added into the flask. The cell suspension was transferred into T25 flask at cell density of 1 x 10^5 cells/ flask. The flask was placed in humidified incubator with 5% CO_2 at 37 °C for 24 hours. The old media was then replaced with 4 mL of fresh complete media.