

**THERAPEUTIC POTENTIALS OF HYPOXIC-  
AND BAICALEIN-ENRICHED FRACTION-  
PRECONDITIONED HUMAN NEURAL STEM  
CELLS FOR *IN VITRO* ISCHEMIC  
STROKE MODEL**

**KANG IN NEE**

**UNIVERSITI SAINS MALAYSIA**

**2018**

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by

**KANG IN NEE**

**Thesis submitted in fulfillment of the requirements**

**for the degree of**

**Doctor of Philosophy**

**April 2018**

## ACKNOWLEDGEMENTS

On the successful completion of this project, I would like to express my sincere gratitude to my main supervisor, Dr. Tan Suat Cheng for her scientific guidance throughout the experimental work and continuous encouragement during the writing of this thesis.

Heartfelt thanks to my co-supervisor, Prof. Dr. Shaharum Shamsuddin for providing the equipment, cell lines and reagents for molecular work. Thank you for the advice and support throughout my studies. I would also like to express my gratitude to my co-supervisor, Dr. Lee Chong Yew for accepting me to conduct part of my research in his laboratory. Thank you for helping me out during the difficult times, for sharing his vast knowledge of phytochemistry and chromatography. It was a pleasant opportunity to work with him.

I am very grateful to all former and present lab members from molecular biology and cell culture laboratories for their help and time throughout my study. I would also like to thank Associate Prof. Dr. See Too Wei Cun and his postgraduate students for sharing chemicals, equipment and knowledge during my difficult times. In addition, I would also like to show my gratitude to all lab members from Prof. Chan Kit Lam's laboratory for sharing their knowledge, experiences, equipment and solvents throughout my work in USM Penang. On top of that, I would also like to extend my gratitude to all the great peoples I have met along the way, particularly to my former colleagues from Cancer Research Initiatives Foundation (CARIF). They have developed my research, analytical and problem solving skills during my 3 years of

tenure with the organisation. Thank you for the friendship and memories.

Furthermore, I am also grateful to the Ministry of Higher Education Malaysia for providing financial aid via MyBrain15 studentship and Research University Individual (RUI) Grant (Grant no: 1001/PPSK/812140). Besides that, I am very grateful to Next Gene Scientific Sdn. Bhd. for allowing me to use the demo unit of CytoSMART™ Lux 2 for a month.

Last but not least, my deepest gratitude goes to my late father who passed away from intracranial hemorrhage, for his continuous encouragement. I always knew that he believed in me and wanted the best for me. Also to my mother and sisters, who have been accompanying me throughout my postgraduate studies. Thank you for staying up late with me in the laboratory while I worked late. The journey would not have been possible without their support.

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## LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

ACTB	$\beta$ -actin
ARE	Antioxidant response element
ATP	Adenosine triphosphate
ATRA	All- <i>trans</i> retinoic acid
ANGPT1	Angiopoietin-1
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CBP	CREB-binding protein
cDNA	Complementary deoxyribonucleic acid
CSL	CBF1/Su(H)/Lag-1
CV	Coefficient of variance
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EtBr	Ethidium bromide
eIF4A	Eukaryotic initiation factor 4A
ERK	Extracellular signal-receulated kinase
ETC	Electron transport chain
FADH <sub>2</sub>	Flavin adenine dinucleotides
FBS	Fetal bovine serum
FIH-1	Factor inhibiting HIF-1

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GPx	Glutathione peroxidase
HIF	Hypoxia-inducible factor
HPLC	High performance liquid chromatography
HPRT1	Hypoxanthine phosphoribosyl transferase 1
HRE	Hypoxia response element
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IVIS	<i>In vitro</i> ischemic stroke
IPO8	Importin 8
KEAP 1	Kelch-like ECH-associated protein 1
MAPK	Mitogen-activated protein kinase
MPTP	Mitochondrial permeability transition pore
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NICD	Notch 1 intracellular domain
Notch 1	Neurogenic locus notch homolog protein 1
Nrf2	Nuclear factor erythroid 2-related factor 2
NSC	Neural stem cell
OD	Optical density
OG	Oxoglutarate
OGD/R	Oxygen-glucose deprivation/reperfusion
<i>O. indicum</i>	<i>Oroxylum indicum</i>
OXPHOS	Oxidative phosphorylation

PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PHD2	Prolyl hydroxylase 2
PKC	Protein kinase C
PPIA	Peptidyl-prolyl isomerase A
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real-time polymerase chain reaction
RAM	RBPJ-associated molecule
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPL13A	Ribosomal protein L13A
RPLP1	60S ribosomal protein large P1
r-tPA	Recombinant tissue plasminogen activator
TUBB3	Tubulin $\beta$ 3 class III
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of means
SGZ	Subgranular zone
SOD	Superoxide dismutase
SVZ	Subventricular zone
TBP	TATA box binding protein
TCA	Tricarboxylic acid
TEMED	Tetramethylenediamine
TLC	Thin layer chromatography
UV	Ultraviolet

VDAC	Voltage-dependent anion-selective channel
VEGFA	Vascular endothelial growth factor A
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
$\alpha$	Alpha
NH <sub>2</sub>	Amine
<i>et al.</i>	And others
$\beta$	Beta
bp	Base pair
Ca	Calcium
Cu	Copper
CO <sub>2</sub>	Carbon dioxide
COOH	Carboxyl
coQ	Coenzyme Q
Cyt c	Cytochrome c
Da	Dalton
°C	Degree celcius
$\Delta$	Delta
E	Efficiency of primer
Fe	Iron
$\gamma$	Gamma
g	Gram
h	Hour

HCl	Hydrochloric acid
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
·OH	Hydroxyl radical
IgG	Immunoglobulin G
κ	Kappa
k	Kilo
L	Litre
μ	Micro
m	Milli
min	Minute
M	Molar
n	Nano
N <sub>2</sub>	Nitrogen
N	Normality
O <sub>2</sub>	Oxygen
pH	Potential of hydrogen
K	Potassium
Psi	Pound of force per square inch of area
p	Probability
Pro	Proline
Akt	Protein kinase B
H <sup>+</sup>	Proton
®	Registered
<sup>1</sup> O <sub>2</sub>	Singlet oxygen
O <sub>2</sub> <sup>•-</sup>	Superoxide anion



s	Second
Na	Sodium
NaOH	Sodium hydroxide
MTT	Tetrazolium
C <sub>T</sub>	Threshold cycle
™	Trademark
Tie-2	Tyrosine kinase-2
vs	Versus
V	Volt
v/v	Volume/volume
w/v	Weight/volume
X g	Times gravity
Zn	Zinc

**POTENSI TERAPEUTIK SEL STEM NEURAL MANUSIA ARUHAN  
SECARA HIPOKSI DAN FRAKSI KAYA DENGAN *BAICALEIN* UNTUK  
MODEL STROK ISKEMIA *IN VITRO***

**ABSTRAK**

Strok iskemia merupakan punca utama kematian di Malaysia selepas penyakit jantung dan barah. Kaedah rawatan biasa tidak mampu memulih dan menjana semula tisu otak pesakit yang mati. Sel stem neural dewasa dalam otak juga tidak mampu memulih tisu tersebut berikutan kadar penjaanaan sel neuron yang terhad gagal membentuk jaringan saraf. Oleh yang demikian, pesakit strok berisiko tinggi untuk serangan semula. Kaedah alternatif berasaskan terapeutik adalah penting untuk meningkatkan pemulihan strok. Mutakhir ini, transplantasi sel stem neural manusia telah menjadi pendekatan terbaik untuk rawatan penyakit neurodegeneratif termasuk strok. Namun demikian, potensi transplantasi sel stem neural adalah terhad kerana kegagalan sel tersebut untuk bertahan dalam kawasan iskemia. Kajian ini bertujuan untuk mengatasi kelemahan tersebut dengan meningkatkan potensi terapeutik sel stem neural melalui aruhan secara hipoksi dan fraksi kaya dengan *baicalein* (F5). Hipoksia paras fisiologi (2% O<sub>2</sub>) selama 24 jam meningkatkan proliferasi, ketahanan dan keupayaan membeza kepada pelbagai jenis sel neural matang. Gen rujukan protein ribosom sub unit besar P1 (*RPLP1*) dan protein ribosom sub unit besar L13A (*RPL13A*) adalah sesuai untuk normalisasi data tindak balas berantai polimerase kuantitatif (qPCR) sel stem neural aruhan secara normoksi dan hipoksi. Aruhan hipoksia mengaktifkan isyarat perlindungan dalam sel melalui transkripsi faktor pendorongan hipoksia-1 alfa (*HIF-1α*), faktor pertumbuhan endotelial vaskular A (*VEGFA*), angiopoietin 1 (*ANGPT1*), protein homolog 1 notch lokus neurogenik

(*Notch 1*), faktor berkait 2-eritroid faktor nukleus 2 (*Nrf2*) and dismutasi natrium (*SOD1*). F5 dipilih sebagai agen aruhan kerana mengandungi *baicalein* yang merupakan penstabil HIF-1 $\alpha$  mimik aruhan hipoksi walaupun dalam keadaan normoksia. Sel stem neural diaruh dengan 1.56  $\mu\text{g}/\text{mL}$  F5 selama 24 jam juga meningkatkan proliferasi, ketahanan dan potensi sel stem neural untuk menjana sel otak matang. Hipoksanthin fosforibosil transferas (*HPRT1*) dan *RPL13A* adalah gen rujukan paling stabil bagi sel stem neural kawalan (0.1% DMSO) dan aruhan secara F5. Seperti hipoksia, aruhan F5 mendorong kepada pengaktifan *HIF-1 $\alpha$* , *VEGFA*, *ANGPT1*, *Notch 1*, *Nrf2* and *SOD1*. Sel stem neural aruhan secara hipoksi dan F5 digunakan untuk merawat model strok iskemia *in vitro* (IVIS). Aruhan F5 menunjukkan kelebihan pertumbuhan lebih pantas menghampiri model IVIS berbanding aruhan hipoksia. Faktor pelindung berpontesi yang dirangsang oleh aruhan hipoksia mempunyai jangka hayat singkat dalam keadaan beroksigen. Aruhan F5 merangsang faktor perlindungan berpontesi yang stabil tanpa dipengaruhi oleh oksigen. Aruhan secara hipoksi dan F5 telah meningkatkan potensi terapeutik sel stem neural untuk terapeutik strok. Kajian ini menunjukkan bahawa sel stem neural diaruh F5 terbukti mempunyai potensi terapeutik yang tinggi, di samping sesuai digunakan secara klinikal untuk rawatan strok iskemia pada masa hadapan.

**THERAPEUTIC POTENTIALS OF HYPOXIC- AND BAICALEIN-  
ENRICHED FRACTION-PRECONDITIONED HUMAN NEURAL STEM  
CELLS FOR *IN VITRO* ISCHEMIC STROKE MODEL**

**ABSTRACT**

Ischemic stroke is the third leading cause of death in Malaysia, closely after heart disease and cancer. Standard treatments for stroke are not totally efficient to repair and regenerate the damaged brain tissue and there are possibilities for the recurrence. Replacement by endogenous adult neural stem cells (NSCs) during ischemic stroke was insufficient to repair injury site due to low neuronal turnover that could integrate into functional neuron network. Therefore, it is imperative to develop alternative therapeutic strategies to improve stroke recovery. Recently, human NSC grafting has emerged as encouraging approach for treating stroke. Nonetheless, the therapeutic potential of NSC-based treatment is limited, mainly due to a large number of implanted cells died after grafting into the injury site. To circumvent this problem, this study aimed to enhance therapeutic potentials of human NSCs prior to transplantation through hypoxic and baicalein-enriched fraction (F5) preconditioning. Hypoxic preconditioning under 2% O<sub>2</sub> for 24 h enhanced NSC self-renewal, survival and multipotency. 60S ribosomal protein large P1 (*RPLP1*) and ribosomal protein L13A (*RPL13A*) were the most reliable reference genes for qPCR normalization of normoxic- and hypoxic-preconditioned NSCs. Hypoxic preconditioning induced innate neuroprotective signaling through transcriptional activation of hypoxia-inducible factor-1 alpha (*HIF-1α*), vascular endothelial growth factor A (*VEGFA*), angiopoietin 1 (*ANGPT1*), neurogenic locus notch homolog protein 1 (*Notch 1*), nuclear factor erythroid 2-related factor 2 (*Nrf2*) and sodium

dismutase 1 (*SOD1*). Based on the HIF-1 $\alpha$  stabilization potential of baicalein at ambient conditions, F5 was postulated to trigger effects mimic hypoxic preconditioning under normoxia. Interestingly, preconditioning with 1.56  $\mu\text{g}/\text{mL}$  of F5 for 24 h increased NSC proliferation, viability and lineage specific differentiation. Hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) and *RPL13A* were the most stably expressed reference genes for qPCR normalization of control (0.1% DMSO) and F5-preconditioned NSCs. Moreover, F5 preconditioning stimulated hypoxia-mimetic signaling intrinsically via *HIF-1 $\alpha$* , *VEGFA*, *ANGPT1*, *Notch 1*, *Nrf2* and *SOD1* upregulation. Both hypoxic- and F5-preconditioned NSCs were applied to *in vitro* ischemic stroke (IVIS) model on wound-healing based culture slide for 72 h of live imaging. F5-preconditioned NSCs accelerated migration and homing towards IVIS model over an experimental period of 72 h compared to hypoxic-preconditioned NSCs. The neuroprotective factors induced by hypoxic preconditioning are postulated to degrade rapidly when exposed to oxygen. Contrarily, F5-preconditioned NSCs attained intrinsic neuroprotective mechanisms without compromising their stability under normoxia. In conclusion, both the hypoxic and F5 preconditioning had successfully enhanced therapeutic potentials of NSCs for ischemic stroke. F5-preconditioned NSCs with enhanced therapeutic efficacy was more likely to be applicable in clinical setting and thus could be a promising therapeutic tool for ischemic stroke in the future.

# CHAPTER 1

## INTRODUCTION

### 1.1 Stroke

Stroke, the nation's third leading cause of death, leading to severe and long-term functional disability (Loo and Gan, 2012; Cheah *et al.*, 2016). In Malaysia, most patients had ischemic stroke rather than hemorrhagic stroke (Jaya *et al.*, 2002). Ischemic stroke is characterized by thrombotic or embolic occlusion of cerebral artery, thereby restricts oxygen, nutrients and glucose supplies to an area of the brain (Figure 1.1A). In contrary, hemorrhagic stroke is characterized by rupture of a blood vessel on or within the brain, subsequently causing severe bleeding into brain parenchyma (Figure 1.1B). According to National Health and Morbidity Survey (NHMS), the prevalence of stroke among Malaysians increased between 2006 and 2011 (Cheah *et al.*, 2016). As a result, stroke causes an economic burden on health-care budgets and the whole nation economic development.

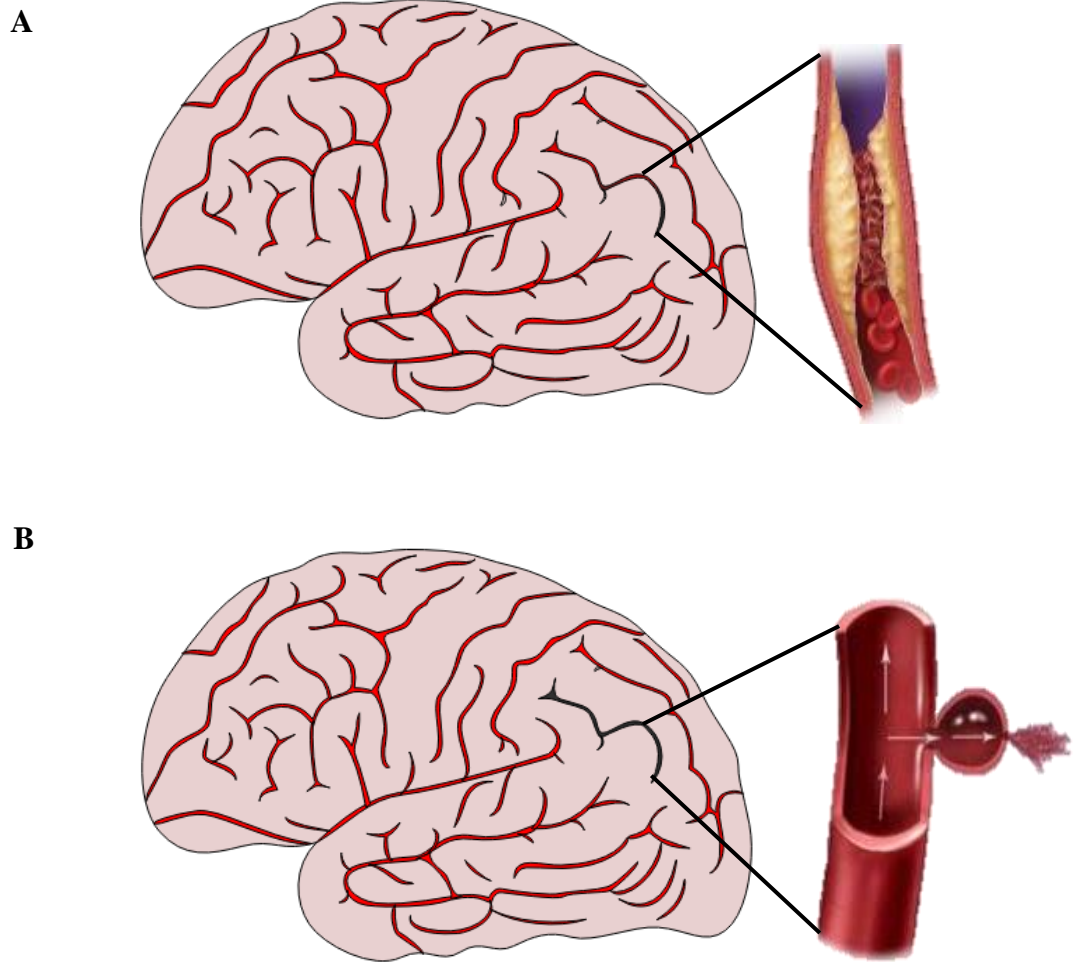


Figure 1.1 A diagram illustrating (A) ischemic stroke and (B) hemorrhagic stroke. Ischemic stroke occurs when a clot blocks blood flow to an area of the brain. Hemorrhagic stroke occurs when blood vessels rupture causing blood leakage into cerebral parenchyma. Figure is generated using Edraw Max software and Microsoft PowerPoint.

### **1.1.1 Pathophysiologic cascades in ischemic stroke**

Ischemic stroke initiates a cascade of pathophysiologic cellular and molecular mechanisms such as energy failure, ionic imbalance, glutamate excitotoxicity, inflammation and free radical overload, resulting in irreversible brain damage (Deb *et al.*, 2010).

#### **1.1.1(a) Energy failure**

Glucose is the primary energy source for the brain (Mergenthaler *et al.*, 2013). Nonetheless, the brain does not store glucose and hence requires a constant supply of glucose (Berg *et al.*, 2002). Due to high energy consumption, abundance mitochondria are present in neurons (Misgeld and Schwarz, 2017). Under physiological conditions, neurons depend mainly on mitochondrial oxidative phosphorylation (OXPHOS) as the main source of adenosine triphosphate (ATP) (Figure 1.2A) (Zheng *et al.*, 2016). This is because OXPHOS generates energy in the form of 36 molecules of ATP for each molecule of glucose (Valvona *et al.*, 2015).

Glucose is metabolized to pyruvate through glycolysis (Valvona *et al.*, 2015). This process also generates ATP and nicotinamide adenine dinucleotides (NADH). Pyruvate enters mitochondria and gets oxidized into acetyl-coA, CO<sub>2</sub> and NADH. Acetyl-coA is subsequently oxidized in tricarboxylic acid cycle (TCA). TCA cycle provide NADH and flavin adenine dinucleotides (FADH<sub>2</sub>) which carry the high-energy electron for OXPHOS at the inner mitochondrial membrane (Lodish *et al.*, 2000). The inner mitochondrial membrane comprises complex I, II, III, IV and V. During OXPHOS, electron transport chain (ETC) passes electrons released from reduced form of NADH and FADH<sub>2</sub> to complex I and II, respectively



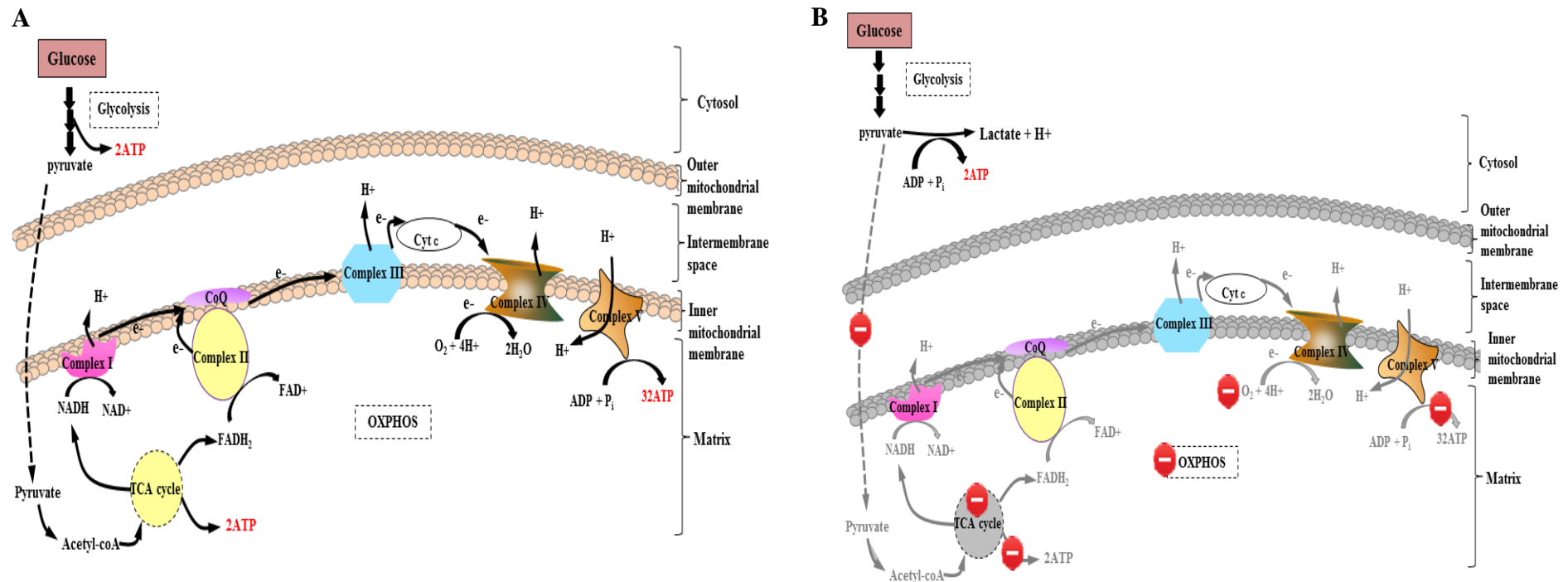


Figure 1.2 An overview of energy metabolism in the brain under (A) physiological conditions and (B) after ischemic stroke. (A) Glucose is converted into pyruvate via glycolysis in the cytosol. Pyruvate is transported into the mitochondria and oxidized to form acetyl-coA. Acetyl-coA is subsequently oxidized via TCA cycle, releasing NADH, FADH<sub>2</sub> and ATP. The electrons transfer from reduced NADH and FADH<sub>2</sub> to oxygen in a series of redox reactions. These reactions released free energy in a form of H<sup>+</sup> gradient across the inner mitochondrial membrane, resulting in ATP synthesis via chemiosmosis. (B) Due to disrupted blood flow, oxygen and glucose levels eventually drop too low for mitochondrial OXPHOS, causing oxidative stress on mitochondria. To compensate the energy supply, glucose is converted into pyruvate via anaerobic glycolysis to meet the metabolic demands. Pyruvate is then converted into the lactate and H<sup>+</sup>. Nevertheless, these reactions generate only 2 molecules of ATP for each molecule of glucose. Ultimately, anaerobic glycolysis fails to compensate the high energy demand of brain cells, leading to energy exhaustion. High accumulation of lactate and H<sup>+</sup> may also lead to acidosis. Figure is generated using Cell Illustrator 5 and Microsoft PowerPoint.

(Lodish *et al.*, 2000). Electrons are then transferred from both complexes to coenzyme Q (coQ). Coenzyme Q transports electrons to complex III and then to cytochrome c (Cyt c). The ETC ends at complex IV where electrons reduce oxygen in the presence of proton ( $H^+$ ) to water. The electrons transfer across complex I, III and IV is also coupled to  $H^+$  transfer, hence, generating a  $H^+$  gradient across the inner mitochondrial membrane (Lodish *et al.*, 2000). Complex V uses the free energy released from  $H^+$  gradient along with ATP synthase to convert adenosine diphosphate (ADP) and inorganic phosphate ( $P_i$ ) into ATP.

In brain, neurons constantly demand for high glucose to perform basic cellular processes such as protein synthesis and energy production (Bédanger *et al.*, 2011; Niven, 2016). Complete loss of blood flow to the brain for only 5 min is deadly to neurons (Lipton, 1999). During ischemic stroke, interruption of blood flow attributes to oxygen and glucose deprivation (OGD). Upon OGD insults, continuous consumption of oxygen and glucose diminish their availability for mitochondrial OXPHOS and ATP generation (Figure 1.2B). Following that, the glucose metabolism switches from aerobic to anaerobic glycolysis for ATP production until glucose is completely depleted (Solaini *et al.*, 2010). Nevertheless, the efficiency of ATP production by anaerobic glycolysis is much lower compared to mitochondrial OXPHOS in which only 2 molecules of ATP produced for each molecule of glucose under anaerobic conditions. Unfortunately, consumption exceeds production and drives energy failure in the brain. In addition, anaerobic glycolysis also produces  $H^+$  and lactate, lowering the pH and causing rapid intracellular acidosis (Solaini *et al.*, 2010).

### 1.1.1(b) Ionic imbalance

Under physiological conditions, the potassium ion ( $K^+$ ) level has higher concentrations intracellular than extracellular (Figure 1.3A) (Cheng *et al.*, 2013). However, the intracellular levels of sodium ion ( $Na^+$ ) and calcium ion ( $Ca^{2+}$ ) are lower than extracellular (Cheng *et al.*, 2013). Following rapid ATP deficit, activity of ATP-dependent transport systems such as  $Na^+/K^+$ -ATPase,  $K^+/Ca^{2+}$ -ATPase and  $Ca^{2+}$ -ATPase pumps are reduced (Figure 1.3B). As consequence, the  $K^+$  channels open and permit  $K^+$  to move outward, leading to accumulation of extracellular  $K^+$  levels. Hence, the cells are hyperpolarize and change the gradient concentrations of  $Na^+$  and  $K^+$  across the plasma membrane (Reading and Isbir, 1980). The  $Na^+$  channels close and retain  $Na^+$  intracellular, leading to accumulation of  $Na^+$  in the cells and depolarization of the membrane potential. Hyperpolarized cells also activate voltage-gated calcium channels which lead to  $Ca^{2+}$  influx.  $Ca^{2+}$  is an intracellular messenger participating in many cellular processes, particularly in signal transduction pathways (Hofer and Lefkimmiatis, 2007). Overload of  $Ca^{2+}$  in the cell further contributes to collapse of membrane potential. Ionic imbalances also induce transient osmotic gradients in the brain as water influx towards area of high  $Na^+$  levels, resulting in cell swelling and edema (Rungta *et al.*, 2015).

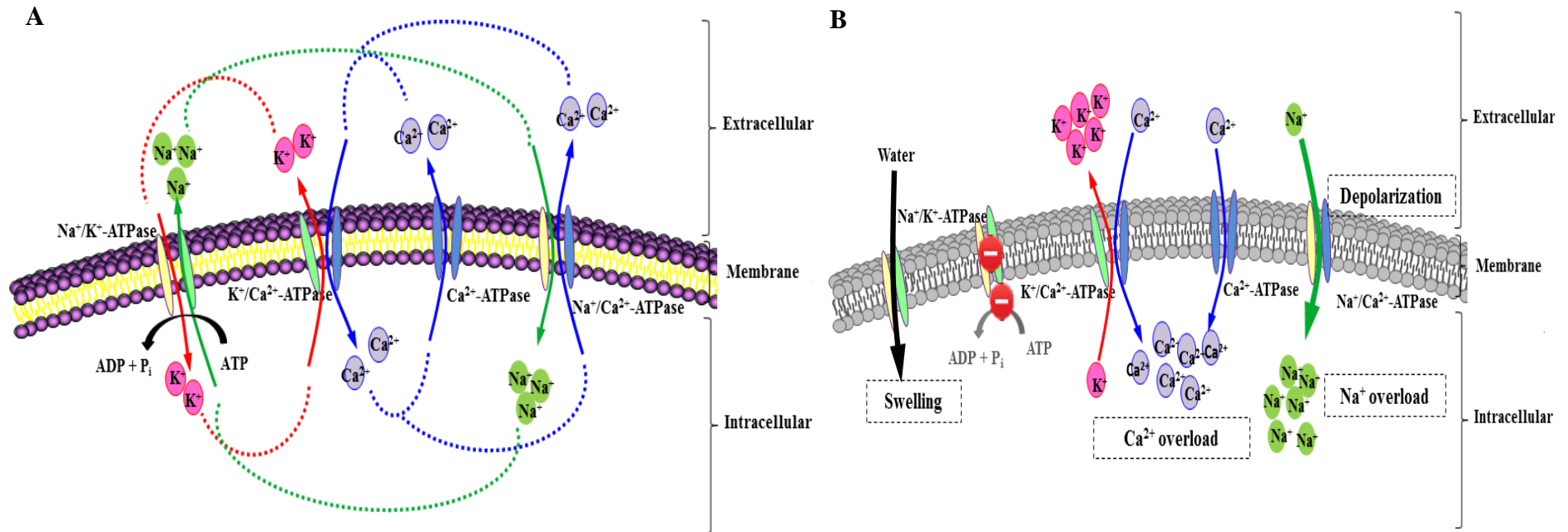


Figure 1.3 An overview of cellular ion homeostasis under (A) physiological conditions and (B) after ischemic stroke. (A) Under physiological conditions,  $\text{Na}^+/\text{K}^+-\text{ATPase}$  uses ATP to pump out 3  $\text{Na}^+$  and take in 2  $\text{K}^+$ . The ionic balance is maintained by  $\text{K}^+/\text{Ca}^{2+}-\text{ATPase}$  which pumps out the  $\text{K}^+$  and takes in  $\text{Ca}^{2+}$ .  $\text{Na}^+/\text{Ca}^{2+}-\text{ATPase}$  maintains the intracellular  $\text{Ca}^{2+}$  by pumping out  $\text{Ca}^{2+}$  and takes in  $\text{Na}^+$ . Hence, the  $\text{K}^+$  level is higher intracellular whereas  $\text{Na}^+$  and  $\text{Ca}^{2+}$  levels are lower intracellular. (B) Due to disrupted blood flow, oxygen and glucose levels eventually drops too low for energy metabolism, causing rapid declination of ATP. As a consequence, ATP-dependent transport systems such as  $\text{Na}^+/\text{K}^+-\text{ATPase}$ ,  $\text{K}^+/\text{Ca}^{2+}-\text{ATPase}$  and  $\text{Ca}^{2+}-\text{ATPase}$  pumps fail to distribute respective ions across the membrane, leading to ionic imbalance. The effects of changes in ion concentration gradient promote depolarization of membrane potential and cell swelling. Figure is generated using Cell Illustrator 5 and Microsoft PowerPoint.

### 1.1.1(c) Glutamate excitotoxicity

Neurotransmitters are chemical messengers that enable excitatory or inhibitory neurotransmission to pass an electrical or chemical signal from a neuron to a target neuron across the synapse (Deutch, 2013). Glutamate is an amino acid-based excitatory neurotransmitter which constitutes for over 90% of the synapses in human brain (Hofmeijer and van Putten, 2012). It is a non-essential amino acid derived from reductive amination of a TCA cycle intermediate,  $\alpha$ -ketoglutarate (Schousboe *et al.*, 2014). Alternatively, glutamate can be synthesized from hydrolysis of glutamine by phosphate activated glutaminase in presynaptic neuron or surrounding glial cells (Schousboe *et al.*, 2014). Under physiological conditions, glutamate is cleared from the synaptic cleft by  $\text{Na}^+$ -coupled glutamate transporters on the plasma membrane of presynaptic terminal and glial cells. In the presynaptic terminal, glutamate is stored in vesicles at the axon (Figure 1.4A). In glial cells, glutamate is converted to glutamine by glutamine synthase in the presence of ATP before being transported back to presynaptic terminal. The glutamine is then recycled for glutamate synthesis in the presynaptic terminal. However, under OGD conditions, lack of ATP production and impairment of  $\text{Na}^+/\text{K}^+$ -ATPase increase intracellular  $\text{Na}^+$  and extracellular  $\text{K}^+$ , which facilitate release of glutamate from the synaptic vesicles into the synaptic cleft (Grewer *et al.*, 2008). Excess accumulation of extracellular glutamate over-activates the glutamate receptors. Following excitatory stimulation, massive extracellular  $\text{Ca}^{2+}$  enters the postsynaptic neuron through ionotropic glutamate receptors such as N-methyl-D-aspartate receptors (NMDARs), leading to phospholipases and proteases accumulation which perturbed the cellular integrity (Figure 1.4B). Ultimately, glutamate excitotoxicity triggers depolarization of postsynaptic neuron, leading to neuronal death (Grewer *et al.*, 2008).

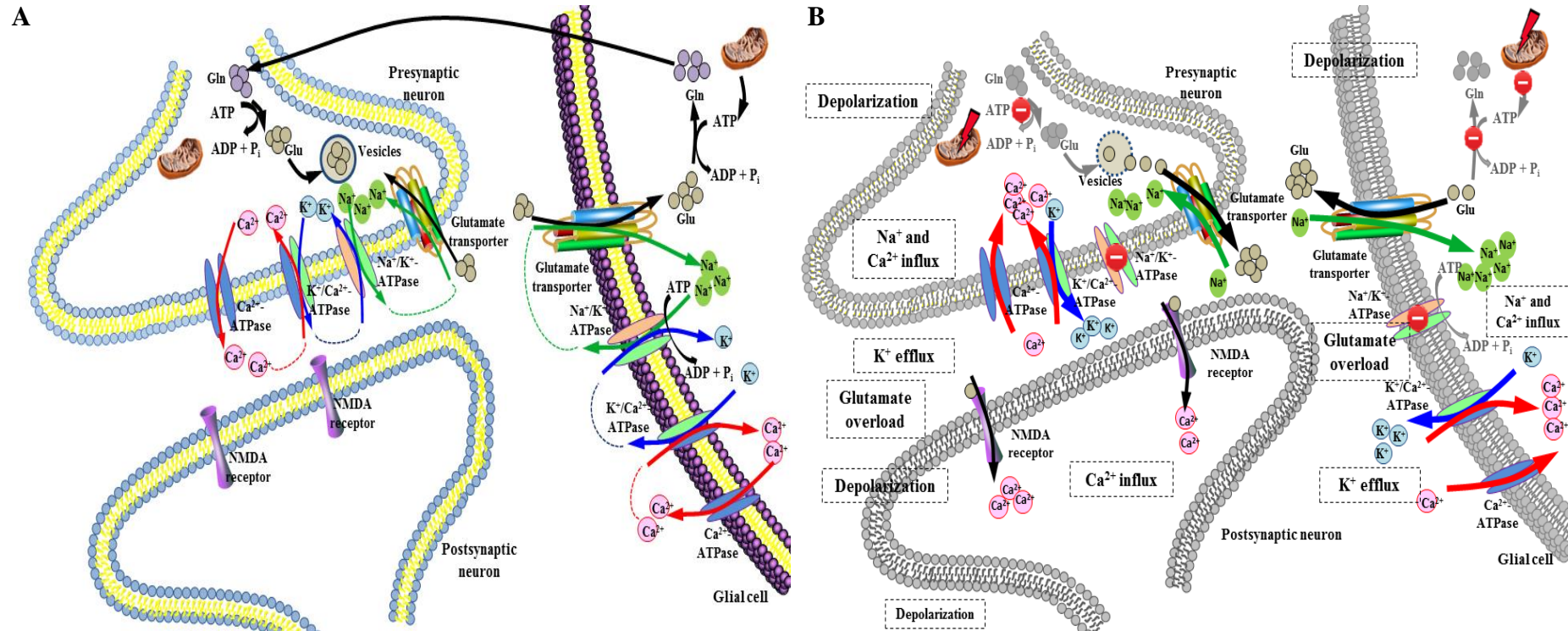


Figure 1.4 Glutamate homeostasis of presynaptic and postsynaptic neurons under (A) physiological conditions and (B) ischemic stroke onset. (A) Under basal condition, glutamate transporters remove glutamate (Glu) from extracellular space and stored in vesicles. In glial cells, glutamate is converted to glutamine (Gln) by glutamine synthase in the presence of ATP before being transported back to presynaptic terminal. The glutamine is then recycled for glutamate synthesis in the presynaptic terminal. The  $K^+$  level is maintained at higher level intracellular.  $Na^+$  and  $Ca^{2+}$  levels are maintained at lower levels intracellular. (B) Under pathological conditions, lack of ATP production and impairment of  $Na^+/K^+$ -ATPase increase intracellular  $Na^+$  and  $Ca^{2+}$  whilst reducing intracellular  $K^+$ , which induce trafficking of glutamate transporters backwards causing glutamate excitotoxicity. Ultimately, depolarization of postsynaptic neuron led to neuronal death. Figure is generated using Cell Illustrator 5 and Microsoft PowerPoint.

#### **1.1.1(d) Inflammation**

The onset of ischemic stroke also initiates various inflammatory cascades by inflammatory cells such as leucocytes, endothelial cells, astrocytes and microglia to produce pro-inflammatory cytokines and adhesion molecules (Lambertsen *et al.*, 2012). Circulating leucocytes then migrate and accumulate in the infarcted lesion before secreting pro-inflammatory mediators which trigger additional injury on neighboring viable cells surrounding the lesion (Vexler *et al.*, 2006). Microglial cells are resident immune cells in the brain (Lull and Block, 2010). Activation of microglia leads to morphologic transformation into phagocytes, subsequently exerting macrophagic functions including phagocytosis, secretion of pro-inflammatory cytokines and generation of reactive oxygen species (ROS) (Lull and Block, 2010). Under pathological conditions, pro-inflammatory cytokines promote the infiltration of inflammatory cells from the circulation into the tissues (Dinarello, 2009). In addition, accumulation of pro-inflammatory cytokines also promotes inflammatory injury through glutamate-mediated excitotoxicity (Olmos and Lladó, 2014).

#### **1.1.1(e) Free radical production**

Normally, mitochondrial OXPHOS converts oxygen molecules into ATP and water. Occasionally, mitochondrial ETC may leak electrons directly to oxygen, resulting in free radical formation. Besides mitochondrial ETC, several cytosolic enzymes such as xanthine oxidase (XO) also contribute to free radical production (Görlach *et al.*, 2015). Free radicals are oxygen-derived oxidants known as ROS. ROS produced in mitochondrial OXPHOS include hydroxyl radical ( $\cdot\text{OH}$ ), superoxide anion ( $\text{O}_2^{\cdot-}$ ), singlet oxygen ( $^1\text{O}_2$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ )

(Halliwell and Gutteridge, 2015). As illustrated in Figure 1.5A,  $O_2^{\cdot-}$  is released from complex I and II into the matrix during electron transport. In addition, complex III also generates  $O_2^{\cdot-}$  within intermembrane space. The latter could be transported through outer mitochondrial membrane by voltage-dependent anion-selective channel (VDAC).

Under physiological conditions, ROS is maintained at low levels by intrinsic antioxidant systems such as catalases, superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Figure 1.5A). In cytosol and intermembrane space of mitochondria, SOD1 converts  $O_2^{\cdot-}$  into  $H_2O_2$  and water. In mitochondrial matrix, SOD2 is responsible for the  $O_2^{\cdot-}$  dismutation. The glutathione peroxidase enzyme (GPx) in the matrix and catalase enzyme in the cytosol catalyse reduction of  $H_2O_2$  into water and oxygen.

Upon ischemia, impaired oxygen supply leads to ATP depletion. Due to ongoing cellular metabolism and ineffective rephosphorylation, ATP is degraded to hypoxanthine.  $Ca^{2+}$  influx also activates  $Ca^{2+}$ -dependent protease to oxidize xanthine dehydrogenase (XDH) to XO,  $O_2^{\cdot-}$  and  $H_2O_2$ . However, XO is unable to convert hypoxanthine to xanthine in the absence of oxygen, leading to accumulation of hypoxanthine.  $H_2O_2$  is partially reduced to highly damaging  $\cdot OH$ . In addition, impaired oxygen supply causes heme proteins to release ferrous cation ( $Fe^{2+}$ ). The  $Fe^{2+}$  reacts with  $H_2O_2$  at higher affinity, giving rise to  $\cdot OH$  through stepwise reduction of Fenton reaction. Overwhelmed ROS can cause opening of the mitochondrial permeability transition pore (MPTP) which irreversibly damage the respiratory chain (Kalogeris *et al.*, 2014). Consequently, intrinsic antioxidant systems



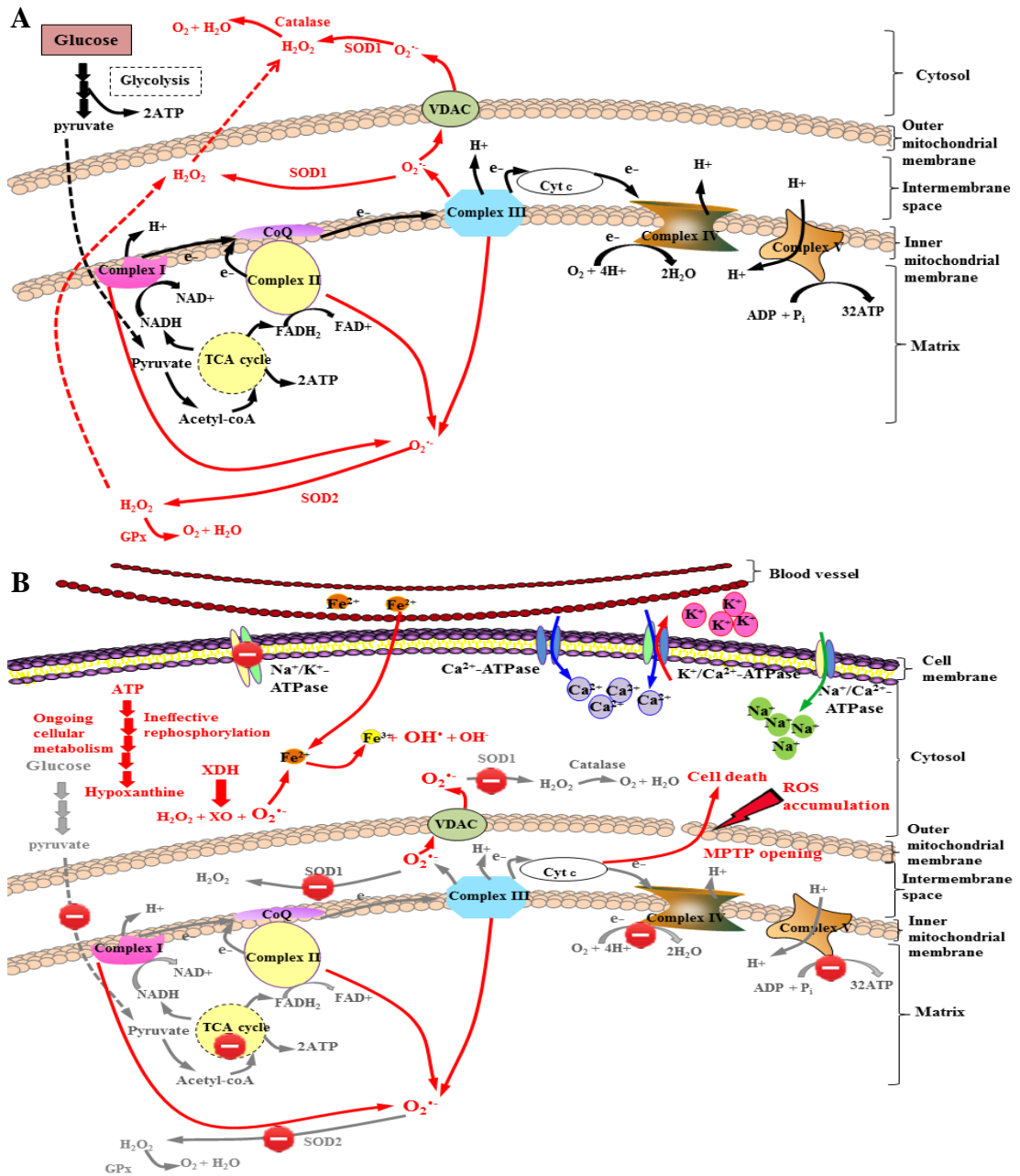


Figure 1.5 Schematic illustration of free radical production and scavenging under (A) physiological conditions and (B) after ischemic stroke as indicated in red. (A) Under normal conditions, complex I, II and III release  $O_2^{\cdot-}$  as byproduct of ETC. Intrinsic antioxidant systems such as SOD1 and SOD2 convert  $O_2^{\cdot-}$  into  $H_2O_2$  and water. Then, catalase and GPx convert  $H_2O_2$  into oxygen and water. Thus, ROS is maintained at low levels. (B) Under ischemia, impaired oxygen and glucose supplies cause ATP depletion. Consequently, ATP is degraded to hypoxanthine.  $Ca^{2+}$  influx activates  $Ca^{2+}$ -dependent proteases which convert XDH to XO,  $O_2^{\cdot-}$  and  $H_2O_2$ . Due to oxygen deprivation, XO is unable to convert hypoxanthine to xanthine.  $H_2O_2$  is partially reduced to highly reactive  $\cdot OH$ . Degraded heme proteins release  $Fe^{2+}$  which react with  $H_2O_2$  to form highly reactive  $\cdot OH$ . ROS accumulation leads to opening of MPTP, Cyt c release, irreversible damage of the respiratory chain, mitochondrial dysfunction and cell death. Figure is generated using Cell Illustrator 5 and Microsoft PowerPoint.

are unable to counterbalance the free radical production. The free radicals oxidize the DNA, protein and lipids, which lead to mitochondrial dysfunction and cell death.

### **1.1.2 Reperfusion injury**

During reperfusion, blood supply returns to the ischemic tissue. The reintroduction of oxygen activates XO that has accumulated during ischemia (Granger and Kvietys, 2015). XO catalyzes conversion of hypoxanthine that has built up during ischemia to form xanthine. Xanthine is subsequently oxidized by XO to form uric acid.  $O_2^{\cdot-}$  and  $H_2O_2$  are produced as byproducts of both oxidation processes.  $H_2O_2$  can be converted to highly destructive  $\cdot OH$  by  $Fe^{2+}$ -mediated Fenton reaction and Harber-Weiss reaction (Das *et al.*, 2014). As a consequence, the intrinsic antioxidant defence systems are overwhelmed by the rapid generation of  $O_2^{\cdot-}$  and  $\cdot OH$  which exacerbate the oxidative stress induced by ischemia. Besides, reperfusion also accelerates the neutrophil infiltration and pro-inflammatory mediators release (Schofield *et al.*, 2013). Neutrophils consist of nicotinamide dinucleotide phosphate (NADPH) oxidase then reduces the oxygen to  $O_2^{\cdot-}$ . Hence, neutrophil activation aggravates ROS formation in the reperfused tissue (Schofield *et al.*, 2013). The inflammatory response also causes endothelial and parenchymal cell damage. Thus, reperfusion extends the ischemia-induced injuries, triggering more severe cascades of cell death.

### **1.1.3 Current treatment for stroke**

The only US Food and Drug Administration (FDA)-approved thrombolytic drug to treat ischemic stroke is recombinant tissue plasminogen activator (r-tPA) (Roth, 2011). It is a clot busting drug that must be given to patients within 4.5 h after

stroke onset to restore the blood flow (Roth, 2011). Besides, r-tPA is only eligible for patients age 18-80 years old without prior history of both stroke and diabetes and not under oral anticoagulants prescription (Demaerschalk *et al.*, 2015). Delayed r-tPA administration after 4.5 h could lead to detrimental side effects such as intracerebral hemorrhagic transformation (HT) and blood brain barrier (BBB) rupture which contributes to high morbidity in patients (Wang *et al.*, 2015). r-tPA cleaves the zymogen plasminogen to form activated plasmin which degrades the fibrin clot (Chevilley *et al.*, 2015).

Neuroendovascular procedures can be done within 6 h of first stroke symptoms and only after r-tPA administration provide better outcomes in patients (Asil and Gultekin, 2016). During neuroendovascular procedures, a catheter is passed through the groin and navigated to the site of artery occlusion. The clot is then removed by the retrievable catheter. The narrow therapeutic window and strict eligibility criterias greatly impede effectiveness of treatment for majority ischemic stroke patients. Furthermore, none of these treatments have been successfully reversing the effect of ischemic stroke by regenerating new cells to replace the damaged brain tissue. As a consequence, stroke patients are usually associated with poor prognosis and high recurrent rate of stroke.

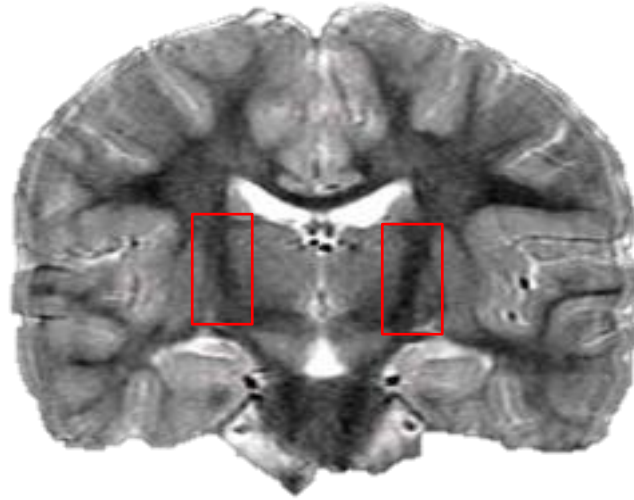
#### **1.1.4 Stem cell therapy**

Since last few years, stem cell therapy has been the most encouraging approach for treating neurodegenerative diseases and stroke (Lunn *et al.*, 2011; Kim *et al.*, 2013; Kumar *et al.*, 2016). The transplanted cells may help to create new circuitry and express factors that protect existing cells (De Feo *et al.*, 2012). Neural stem cells

(NSCs) are considered as the most optimal cell type for stem cell-mediated therapy of brain disorders such as stroke (Kim *et al.*, 2013). This is because they share the same tissue origin of the damaged brain cells and are originally meant to replenish the brain cells *in vivo*. In human brain, NSCs reside in two high-density cell division sites, the subventricular zone (SVZ) and subgranular zone (SGZ) (Figure 1.6) (Gonzalez-Perez, 2012). NSCs are self renewal and able to differentiate into neuron, astrocyte and oligodendrocyte. They also have a lower oncogenic potential and immunological rejection (De Feo *et al.*, 2012). Thus, NSCs provide the great potential for clinical applications.

NSC transplantation has been studied in ischemic stroke animal models and human trial (Englund *et al.*, 2002; Kelly *et al.*, 2004; Date *et al.*, 2006; Jin *et al.*, 2011; Zhang *et al.*, 2011; Muir and Sinden, 2015). Transplanted NSCs have been reported to contribute for neuroprotective effects in ischemic stroke. Several studies have demonstrated that transplanted NSCs could migrate to the ischemic areas, differentiate into mature neurons and even promote endogenous neurogenesis (Jin *et al.*, 2011; Zhang *et al.*, 2011; Mine *et al.*, 2013). Besides, transplanted NSCs could ameliorate cerebral inflammation by reducing macrophage infiltration (Lee *et al.*, 2008). Transplanted NSCs also promote angiogenesis at surrounding infarcted region through release of paracrine factors such as vascular endothelial growth factor A (VEGFA), angiopoietin-1 (ANGPT1), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Hicks *et al.*, 2013). Furthermore, Zhao and Wang (2013) demonstrated the anti-apoptotic effects of transplanted NSCs through enhanced mitogen-activated protein kinase/extracellular receptor kinase (MAPK/ERK) signaling in ischemic brain.

**A**



**B**

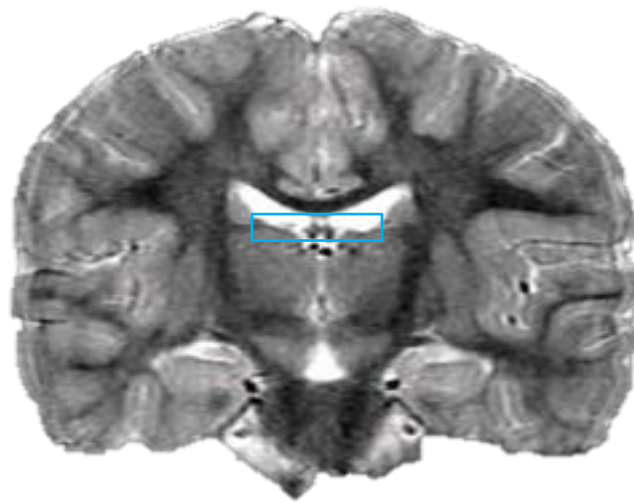


Figure 1.6 Coronal section of human adult brain. (A) NSCs reside within SVZ of the lateral ventricle (red boxes). (B) In adult hippocampus, NSCs are located in the SGZ of the dentate gyrus (blue box). Adapted from DeFelipe (2011).

Despite the great therapeutic potential of NSCs for ischemic stroke, the survival of grafted cells within the infarcted area remains a serious concern for NSC transplantation. This is because the ischemic/reperfused region is very harsh for cell survival due to the excessive free radicals, glutamate excitotoxicity and inflammation. As little as 0.09% retention rate of grafted cells were observed in mice with transient middle cerebral artery occlusion (MCAO) (Bacigaluppi *et al.*, 2009). Low cell survival rate limits the capacity of NSCs to repair the injured area and this poses a much more difficult challenge to the NSC-based therapy for ischemic stroke. To circumvent this problem, preconditioning of NSCs was employed.

## **1.2 Preconditioning strategy**

The concept of preconditioning was first described in the past 30 years, whereby one or more brief episodes of sub-lethal stimuli was introduced to render the endogenous protective mechanisms against subsequent lethal injury (Murry *et al.*, 1986). Preconditioning can be achieved through physiological sub-lethal stimuli such as hypoxia, heat shock and carbon monoxide (Sharp *et al.*, 2004; Harder *et al.*, 2005; Vieira *et al.*, 2008). Besides, preconditioning can also be achieved through pharmacological agents such as minocycline, doxycycline, sevoflurane, cobalt chloride, baicalein and aromatic-tumerone (Wang *et al.*, 2010a; Sakata *et al.*, 2012; Wang *et al.*, 2012; Chang *et al.*, 2013; Dai *et al.*, 2014; Hucklenbroich *et al.*, 2014). This study focused on physiological and pharmacological preconditioning of NSCs using hypoxia and baicalein-enriched fraction from *Oroxylum indicum*, respectively.

### **1.2.1 Hypoxic preconditioning**

Hypoxic preconditioning involves brief periods of hypoxia that triggers various protective signaling pathways and enhances resilience to ischemia (Sharp *et al.*, 2004; Wei *et al.*, 2017). Transcriptional responses to hypoxia are mediated by the hypoxia-inducible factor (HIF) (Sharp *et al.*, 2004). Under hypoxia, activated HIF binds to hypoxia response elements (HREs) and subsequently activates expression of target genes involved in angiogenesis, energy metabolism and cell proliferation. Hypoxic preconditioning has been shown to promote NSC survival after transplantation into ischemic rat brain (Wakai *et al.*, 2016).

Besides, previous studies had reported that NSC microenvironment is hypoxic by nature, whereby the oxygen level in the brain may be as low as 2% (Studer *et al.*, 2000). Thus, *in vitro* cultivation of NSCs in a typical incubator condition of 21% oxygen does not reflect the physiologic norms. The normoxic condition is unlikely to be optimal for maintaining normal proliferative state of NSCs (Studer *et al.*, 2000). Several studies have demonstrated that priming the endogenous NSCs using hypoxic preconditioning method can enhance the cell proliferation and differentiation in animal models (Giese *et al.*, 2010; Wakai *et al.*, 2016).

### **1.2.2 Pharmacological preconditioning by baicalein-enriched fraction**

Pharmacological preconditioning involves brief periods of exposure to pharmacological agents that triggers intrinsic protective mechanisms and enhances resilience to subsequent lethal insults (Gidday, 2010). Baicalein (5,6,7 trihydroxyflavone) is a flavonoid aglycan derived from *Scutellaria baicalensis* and *Oroxylum indicum* (Majeed *et al.*, 2017). Like many flavonoid compounds,

baicalein can exert both antioxidant and pro-oxidant effects depending on cell types (Woo *et al.*, 2005; Kang *et al.*, 2012). Spectroscopic scanning measurements by Cho *et al.* (2008) revealed the binding of baicalein to prolyl hydroxylase 2 (PHD2) and factor inhibiting HIF-1 (FIH-1) under normoxia. Baicalein was found to compete with 2-oxoglutarate (2OG) for the active sites of PHD2 and FIH-1 through its 3-hydroxyl structure. Hence, baicalein abrogated hydroxylation of hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ ) similar to those observed in hypoxic preconditioning. In addition, Chang *et al.* (2013) demonstrated that pharmacological pretreatment of chick cardiomyocytes using baicalein derived from *S. baicalensis* for 10 min prior to ischemia/reperfusion mediated a pro-oxidant preconditioning effects which mimicks hypoxic preconditioning. Consequently, pre-treatment with baicalein conferred cytoprotection against ischemia and reperfusion injury in those cardiomyocytes and reduced apoptosis (Chang *et al.*, 2013). The effects of baicalein on NSC have not been determined previously. Baicalein has been described to exert anti-proliferative and pro-proliferative activity depending on cell types. Baicalein treatment was found to attenuate proliferation of numerous human cancer cell lines (Cathcart *et al.*, 2016; Bie *et al.*, 2017; Zhou *et al.*, 2017). However, it promoted proliferation in hematopoietic stem cells (Patwardhan *et al.*, 2014). Hence, it is crucial to determine the effects of baicalein-enriched fraction particularly on NSCs.

### **1.2.2(a) Baicalein-enriched fraction from *Oroxylum indicum***

Considering the promising neuroprotection potentials of baicalein, this study sought to produce an enriched baicalein fraction from the leaves of *O. indicum* as pharmacological agent for preconditioning. Besides *O. indicum* leaves, baicalein is also the major constituent in the roots of *S. baicalensis* (Aliev *et al.*, 2014). However,



*S. baicalensis* requires destructive harvest which may lead to severe depletion of medicinal plant resources (Nishteswar, 2014). Hence, in this study, *O. indicum* leaves offer better resources without the removal of entire plant.

Briefly, *O. indicum* is a medicinal herb belongs to family of Bignoniaceae that is commonly found in many regions of South-east Asia (Harminder *et al.*, 2011). It is a deciduous soft wooded tree with pinnately compound, ovate-elliptic leaves and acuminate leaf tips (Figure 1.7A and B) (Harminder *et al.*, 2011). This tree also has large flowers with stinky odour that bloom at night time to attract pollinators (Figure 1.7C). It also has long and flattened fruit pods that hang down from the thick branches like dangling swords (Figure 1.7D). When the pod burst, the flat and semi-transparent winged seeds flutter like butterflies (Figure 1.7E). Hence, the seeds are known as “mu hu die” (tree butterfly) in Chinese herbal medicine (Chen *et al.*, 2003b). In Malaysia, it is commonly known as “Beko” and the young leaves as well as fruits are consumed raw as salad by the local community. Different parts of *O. indicum* such as fruits, root barks and leaves have been applied in traditional medicine to treat bronchitis, dyspepsia, asthma, gastropathy, inflammation and microbial infection (Harminder *et al.*, 2011). Preconditioning by baicalein-enriched fraction is also postulated to trigger similar neuroprotective mechanisms induced by hypoxic preconditioning.

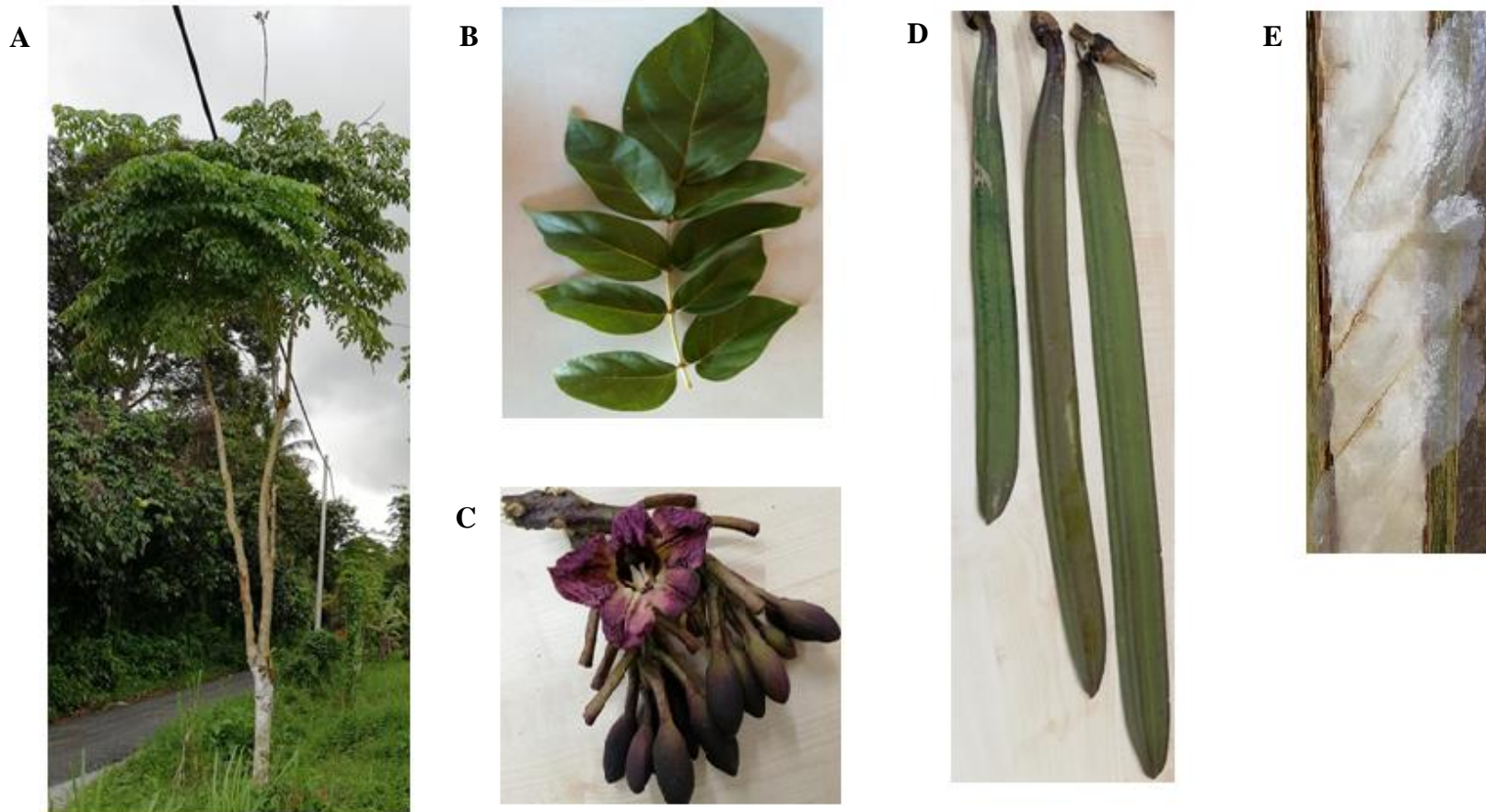


Figure 1.7 Representative images of *O. indicum*. (A) *O. indicum* is a medium-sized tree that branched at top. (B) The leaves are pinnately compound, ovate-elliptic with acuminate tips. (C) The flower emits strong and stinky smell to attract night pollinators. (D) Long and flattened fruit pods that hang down from the thick branches like dangling swords. (E) The seeds are flat with semi-transparent wings. Pictures of plants were taken during botanical field trip for plant specimen collection.

### 1.2.3 Preconditioning-induced neuroprotective signaling

Physiological and pharmacological preconditioning have been reported to enhance resistance to lethal insults through activation of endogenous survival cascades such as HIF-1 $\alpha$ , neurogenic locus notch homolog protein 1 (Notch 1), VEGFA, ANGPT1, nuclear factor erythroid 2-related factor 2 (Nrf2) and SOD1 (Figure 1.8) (Chen *et al.*, 2003a; Belaidi *et al.*, 2006; Stubbs *et al.*, 2012; Zhou *et al.*, 2013; Huang *et al.*, 2014).

#### 1.2.3(a) Hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ )

HIF-1 is a basic Helix-Loop-Helix-Period-Arnt-Single minded-homology (bHLH-PAS) domain transcription factor composed of an alpha ( $\alpha$ ) and a beta ( $\beta$ ) subunit (Semenza *et al.*, 1997). The oxygen-labile  $\alpha$  subunits consisted of HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-3 $\alpha$  whereas  $\beta$  subunit, HIF-1 $\beta$ , is constitutively presented in the nucleus (Semenza *et al.*, 1997). Apart from bHLH-PAS domain, HIF-1 $\alpha$  also comprises of transactivation domains (TAD) and oxygen-dependent degradation domain (ODD) as shown in Figure 1.9A (Semenza *et al.*, 1997).

HIF-1 $\alpha$  expression is regulated by PHD2 and FIH-1. Under normoxic conditions, PHD2 catalyzes hydroxylation of two highly conserved proline residues (Pro402 and Pro564) within the ODD of HIF-1 $\alpha$  (Figure 1.9B). The enzymatic process utilizes Fe<sup>2+</sup> as co-factor whilst oxygen and 2OG as substrates. An E3 ubiquitin ligase complex known as von Hippel-Lindau (pVHL) further targets the hydroxylated HIF-1 $\alpha$  for proteasomal degradation. In addition, FIH-1 catalyzes hydroxylation of asparagine residue (Asn803) within the C-TAD of HIF-1 $\alpha$  (Figure 1.9C). Similar to PHD2, the hydroxylation requires Fe<sup>2+</sup> as co-factor whilst oxygen and 2OG as

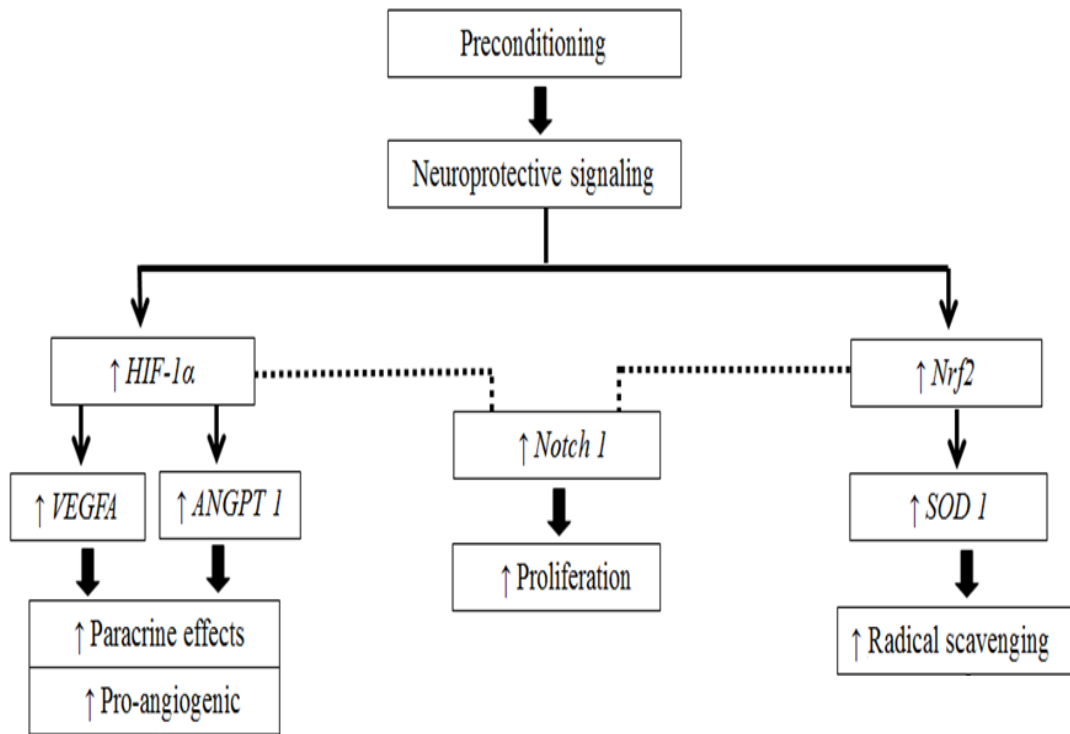


Figure 1.8 Preconditioning-induced neuroprotective signaling. Preconditioning modulated adaptive and recuperative mechanisms through HIF-1 $\alpha$ , Notch 1, VEGFA, ANGPT1, Nrf2 and SOD1. HIF-1 $\alpha$  enhanced VEGFA and ANGPT1 signaling to stimulate paracrine effects and angiogenesis whereas Nrf2-mediated SOD1 expression stimulates free radical scavenging. The crosstalk of HIF-1 $\alpha$  and Nrf2 with Notch 1 promotes cell proliferation.

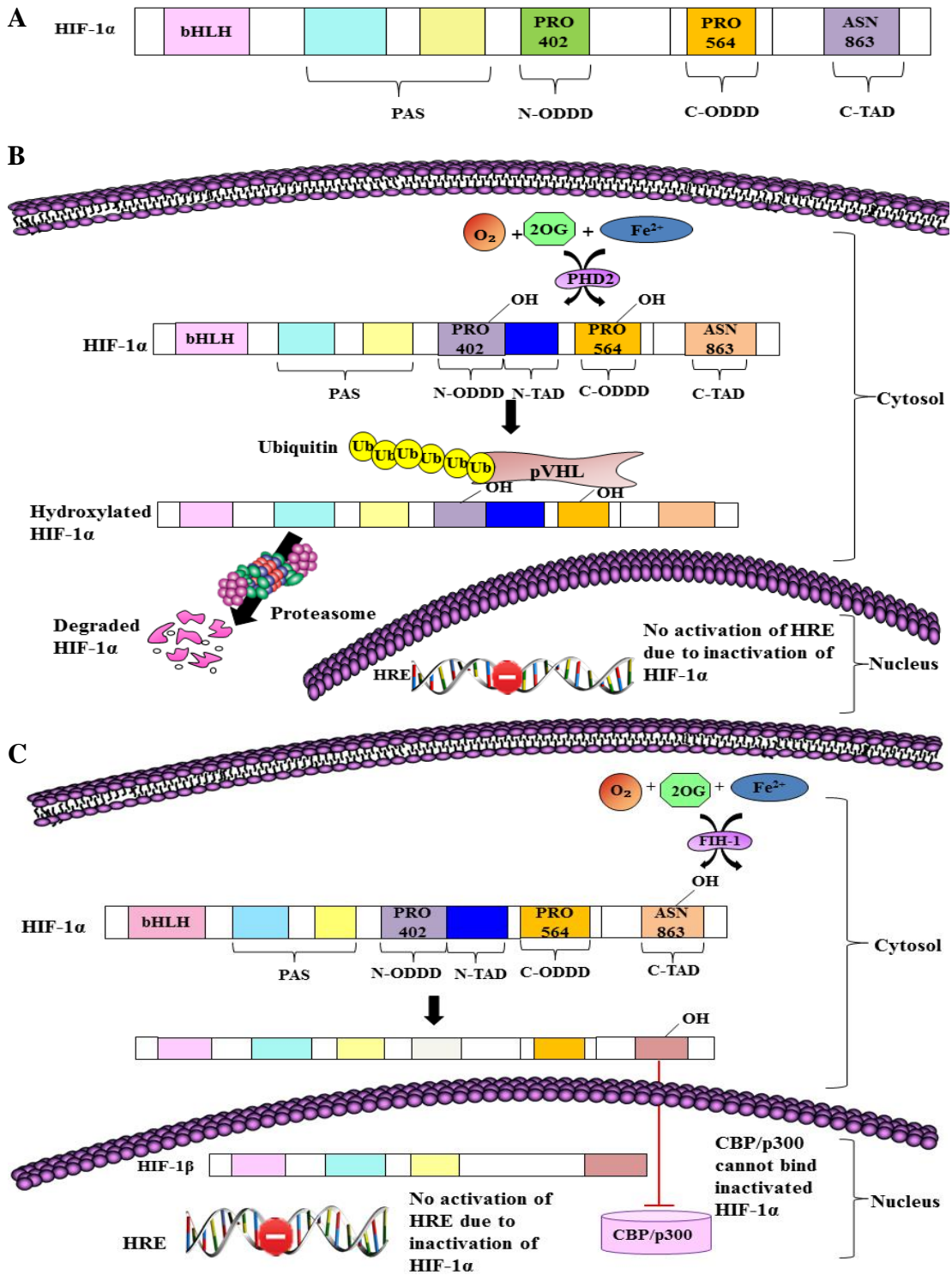


Figure 1.9 Schema illustrating (A) HIF-1 $\alpha$  domain structure, (B) PHD2 and (C) FIH-1 regulated HIF-1 $\alpha$  degradation under normoxic conditions. (A) HIF-1 $\alpha$  contain bHLH-PAS, TAD and ODDD. (B) Using oxygen, 2OG and Fe<sup>2+</sup>, HIF-1 $\alpha$  is degraded by PHD2 signaling. (C) FIH-1 also catalyzes hydroxylation of HIF-1 $\alpha$  in the presence of oxygen, 2OG and Fe<sup>2+</sup>. Hydroxylated HIF-1 $\alpha$  could not bind to CBP/p300 for downstream HIF-1 $\alpha$  target genes. Hence, transcriptional activity of HIF-1 $\alpha$  is inhibited (Masson and Ratcliffe, 2003). Figure is generated using Cell Illustrator 5 and Microsoft PowerPoint.