



Faculty of Medicine and Health Sciences

**Optimisation of PC12 Cell-based *In Vitro* Stroke Model for Screening of
Potential Neuroprotective Agents**

Chua Pin Fen

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Optimisation of PC12 Cell-based *In Vitro* Stroke Model for Screening of
Potential Neuroprotective Agents

Chua Pin Fen

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DECLARATION

I hereby declare that this thesis is my original work. All sources of information which were used as references to compose this thesis have been acknowledged within the text. The thesis has not been accepted for any degree and is not concurrently submitted in candidature for any other degree.

Chua Pin Fen

Molecular Pharmacology Lab

Faculty of Medicine and Health Sciences

Universiti Malaysia Sarawak

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ABSTRACT

Stroke is one of the commonest causes of death and disability worldwide. To date, no neuroprotective agent is approved clinically for treatment of acute ischaemic stroke. PC12 cell-based *in vitro* stroke model has been used widely to screen for neuroprotective agents. The most common approach is to differentiate PC12 cells into a neuronal phenotype and subject the differentiated cells to oxygen glucose deprivation (OGD) to simulate an ischaemic stroke. However, there are increasing reports showing certain PC12 cell variants either poorly or cannot differentiate into neuron-like cells. Additionally, optimisation studies for cell culture media and adhesive substrate for commonly-used PC12 variants are limited. This study aimed to optimise cell culture conditions of three commonly-used PC12 cell variants and test their response to nerve growth factor (NGF)-induced differentiation. PC12 cells (Riken cell bank), PC12 Adh cells and PC12 Neuroscreen-1 (NS-1) cells were studied. Optimisation of cell culture media and adhesive substrates were done by comparing cell morphology in different media and substrates. Adaptation of the three PC12 cell variants to a serum-free supplemented media was necessary because drug intervention studies need to be done in the absence of serum to avoid confounding effects. Comparison of the NGF-induced differentiation for the three PC12 cell variants was done by counting the percentage of neurite-bearing cells. The PC12 variant with the highest response to NGF underwent studies to optimise its duration of OGD. Differentiated-cells were subjected to different durations of OGD followed by measurement of cell viability and enzyme activity in the execution phase of apoptosis (caspase 3 and 7) to determine the optimal duration of OGD for *in vitro* stroke model. The *in vitro* model was validated by treating the OGD-treated cells with a known neuroprotectant, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) for

24 hours. The optimal culture media for PC12 cells (Riken) and NS-1 cells was found to be DMEM high glucose. Collagen IV was the best substrate for PC12 cells (Riken) and NS-1 cells. PC12 Adh cells showed no preference for media or substrates. Both NS-1 cells and PC12 cells (Riken) were successfully adapted to serum-free supplemented media. However, PC12 Adh cells failed to grow in a serum-free supplemented media. NS-1 cells gave the highest NGF-induced differentiation ($72.7 \pm 9.1\%$) followed by PC12 cells (Riken, $36.0 \pm 5.6\%$) and PC12 Adh cells ($6.9 \pm 0.8\%$). NS-1 cells achieved optimal differentiation after three days of 150 ng/mL NGF treatment. The optimal duration of OGD to induce cell injury was three hours. Treatment with 8-OH-DPAT on NGF-differentiated NS-1 cells after three hours of OGD showed a significant reduction in apoptosis ($p < 0.01$, One-way ANOVA followed by post hoc Tukey's test). In conclusion, we have shown that each PC12 variant has differing requirements for media and adhesive substrates. NS-1 cells were the optimal PC12 cell variant for an *in vitro* stroke model due to its high level of NGF-induced differentiation. DMEM high glucose was the optimal media and collagen IV was the best substrate for NS-1 cells. We also provide the first report that 8-OH-DPAT is neuroprotective in NS-1 cells. We conclude that we have optimised and set up an *in vitro* stroke model to screen for potential neuroprotective agents.

Keywords: Stroke, PC12 cell, adhesive substrate, NGF-differentiation, oxygen deprivation assay, apoptosis assay, neuroprotective agents.

Pengoptimuman Model Strok In Vitro berasaskan Sel PC12 untuk Penyaringan Agen Neuroprotektif yang Berpotensi

ABSTRAK

Penyakit Strok adalah salah satu penyebab utama kematian dan kecacatan di seluruh tetapi masih tiada agen neuroprotektif diluluskan untuk rawatan strok iskemia. Model strok in vitro berasaskan sel PC12 telah digunakan secara meluas untuk penyaringan agen-agen neuroprotektif. Pendekatan yang paling lazim digunakan adalah membezakan sel PC12 kepada fenotip neuron dan mendedahkan sel tersebut kepada keadaan kekurangan glukosa dan oksigen (OGD) untuk mensimulasikan strok iskemia. Walau bagaimanapun, terdapat peningkatan laporan yang menunjukkan varian sel PC12 tertentu yang tidak dapat atau kurang berupaya untuk berbeza kepada sel seperti neuron. Kajian pengoptimuman untuk media kultur sel dan substrat perekat untuk varian PC12 yang lazim digunakan adalah terhad. Kajian ini bertujuan untuk mengoptimumkan keadaan kultur sel untuk tiga varian sel PC12 dan menguji tindak balas mereka terhadap pembezaan dengan faktor pertumbuhan saraf (NGF). PC12 (bank sel Riken), PC12 Adh dan PC12 Neuroscreen-1 (NS-1) telah dikaji. Pengoptimuman media kultur sel dan substrat pelekak dilakukan dengan membandingkan morfologi tiga varian sel dalam media dan substrat pelekak yang berbeza. Penyesuaian varian sel tiga PC12 ke media tambahan tanpa serum adalah perlu kerana kajian ubat baru perlu dilakukan tanpa serum untuk mengelakkan kesan yang mengelirukan. Perbandingan tindak balas kepada pembezaan yang disebabkan oleh NGF untuk tiga varian sel telah dilakukan dengan mengira peratusan sel yang mengandungi neurit. Varian sel yang menunjukkan tindak balas tertinggi kepada NGF digunakan untuk mengoptimumkan tempoh OGD. Sel tersebut tertakluk kepada tempoh OGD yang berlainan dan diikuti dengan pengukuran daya aktif sel dan aktiviti enzim dalam fasa pelaksanaan apoptosis (caspase 3 dan 7) untuk menentukan tempoh optimum OGD. Model

strok in vitro disahkan dengan merawat sel yang telah dcedera oleh OGD dengan menggunakan agen neuroprotektif yang dikenali sebagai 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) selama 24 jam. Hasil kajian menunjukkan media kultur optimum untuk PC12 (Riken) dan NS-1 adalah DMEM tinggi gula. Kolagen IV adalah substrat terbaik untuk PC12 (Riken) dan NS-1. PC12 Adh tidak menunjukkan keutamaan dalam mana-mana media atau substrat. NS-1 dan PC12 (Riken) berjaya disesuaikan dengan media tambahan tanpa serum dan dapat hidup di media tersebut. Walau bagaimanapun, PC12 Adh gagal berkembang dalam media tambahan tanpa serum. NS-1 menghasilkan pembezaan yang disebabkan oleh NGF ($72.7 \pm 9.1\%$) yang tertinggi diikuti oleh PC12 (Riken, $36.0 \pm 5.6\%$) dan PC12 Adh ($6.9 \pm 0.8\%$). Sel NS-1 mencapai pembezaan optimum selepas tiga hari rawatan 150 ng/mL NGF. Tempoh optimum OGD untuk merangsang kecederaan sel adalah tiga jam. Rawatan dengan 8-OH-DPAT pada sel NS-1 yang telah dibezakan selepas tiga jam OGD menunjukkan pengurangan apoptosis yang signifikan ($p < 0.01$) (satu arah ANOVA diikuti oleh ujian Tuki post hoc). Kesimpulannya, kami telah menunjukkan bahawa setiap varian PC12 mempunyai keperluan berbeza untuk media dan substrat pelekat. Kami melaporkan bahawa 8-OH-DPAT adalah neuroprotektif dalam NS-1 dengan kali pertama. NS-1 adalah varian sel PC12 yang optimum untuk model strok in vitro kerana mempunyai tahap perbezaan oleh faktor NGF yang paling tinggi berbanding dengan dua varian PC12 yang lain. DMEM tinggi gula adalah media optimum dan kolagen IV adalah substrat terbaik untuk NS-1. NS-1 yang didedahkan kepada tiga jam OGD menyerupai strok iskemia. Kesimpulannya, kami telah mengoptimumkan dan menubuhkan model strok in vitro untuk menyaring agen-agen neuroprotektif yang berpotensi.

Kata kunci: Strok, sel PC12, substrak pelekat, pembezaan dengan faktor pertumbuhan saraf, kekurangan glukosa dan oksigen, apoptosis, agen-agen neuroprotektif

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATCC	American type culture collection
ATP	adenosine triphosphate
BD	Becton Dickinson
BSA	bovine serum albumin
CO ₂	carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
ERK1/2	extracellular signal-regulated kinases
FBS	fetal bovine serum
F-12K	Ham's F-12K (Kaighn's) Medium
HBSS	Hank's balanced salt solution
HCL	hydrochloric acid
HS	horse serum
5-HT1A	5-hydroxytryptamine 1A receptors
mg	milligram

mL	mililitre
mM	milimolar
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
ng	nanogram
NGF	nerve growth factor
NIH	National Institutes of Health
nM	nanomolar
NS-1	Neuroscreen-1
OGD	oxygen glucose deprivation
OGD/R	oxygen glucose deprivation/reperfusion
8-OH-DPAT	8-hydroxy-2(di-n-propylamino) tetralin
PBS	phosphate buffered saline
PC	pheochromocytoma
rpm	rotation per minute
RPMI	Roswell Park Memorial Institute
RTKs	receptor tyrosine kinase
SD	standard deviation

Trk	tyrosine kinases
μL	microlitre
μg	microgram
μM	micromolar
%	percentage
°C	degree Celcius

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Stroke

Stroke is the second commonest cause of death worldwide (Katan & Luft, 2018). Its global burden is high, inclusive of increasing mortality, incidence, disability-adjusted life year and economic impact, particularly in low- and middle-income countries (Mukherjee & Patil, 2011). It can cause cognitive and functional disabilities, contributing significantly to long term health care costs (Azad, Veeravagu, & Steinberg, 2016). In Malaysia, stroke is the third leading causes of death (Department of Statistic Malaysia, 2018). The incidence of local ischaemic stroke is on the trend of rising annually by 29.5% (Lee, Shafie, Sidek & Aziz, 2017). The prevalence of stroke was reported as 0.7%, with 1.7% among those aged 55-59 years, 2% in 60-64 years, 3% in 65-69 years, 3.5% in 70-74 years and 7.8% in 75 years and beyond in the fourth National Health and Morbidity Survey (NHMS) in 2011 (Ministry of Health Malaysia, 2011). Prognosis of the disease depends on many factors including age, stroke severity, infarct location, stroke mechanism, clinical findings and related complications. In addition, medical interventions for example thrombolysis, thrombectomy, stroke unit care, and rehabilitation influences the outcome of the disease.

1.1.1 Pathogenesis of Stroke

Stroke are caused by ischaemia (loss of blood supply) or haemorrhage (bleeding). Around 80% of stroke are caused by ischaemia. The most common ischaemic strokes include

thrombotic stroke and embolic stroke. A thrombotic stroke occurs when a blood clot (thrombus) forms in one of the arteries that supply blood to your brain. A clot may be caused by fatty deposits (plaque) that build up in arteries and cause reduced blood flow (atherosclerosis) or other artery conditions. An embolic stroke occurs when a blood clot or other debris forms away from your brain (commonly in your heart) is swept through your bloodstream to lodge in narrower brain arteries. This type of blood clot is called an embolus.

In ischaemic stroke, cerebral blood flow is disrupted throughout the affected region of the brain. Blood flow disruption to the tissue is not uniform due to the presence of collateral circulation. Consequently, a “core” region of infarction develops in which blood flow is severely reduced and tissue undergoes necrosis within minutes (Woodruff et al., 2011) as insufficient adenosine triphosphate (ATP) is present to maintain homeostatic ionic gradients and metabolic functions (Astrup, Symon, Branston, & Lassen, 1977). Surrounding this core region in which blood flow is severely reduced is the penumbra, a tissue distribution in which flow is less severely reduced (Astrup et al., 1977). Penumbra is a brain tissue area that is affected during focal ischaemia but brain cells are not yet dead (Lo, 2008) and are salvageable (Khoshnam, Winlow, Farzaneh, Farbood, & Moghaddam, 2017). Over the past decade, research has shown that neurons in the penumbra area undergo apoptosis after several hours or days (Broughton, Reutens, & Sobey, 2009). In contrast to the core region delayed mode of cell death (apoptosis) (Mehta, Manhas, & Raghubir, 2007). Hence, the existence of penumbra plays a fundamental role in current treatment approach on ischaemic stroke (Jung et al., 2013; Moskowitz, Lo, & Iadecola, 2010).

The pathophysiology of stroke is complex and involves many processes. Ischaemia of the neurons causes energy failure (ATP depletion). This leads to failure of Na^+ and K^+ pump, neuronal depolarization and release of glutamate (Puig, Brenna, & Magnus, 2018). Activation of specific glutamate receptors markedly increases intracellular Ca^{2+} and Na^+ . Subsequently K^+ is released into the extracellular space. Edema occurs when the water shifts to the intracellular space. Increased levels of intracellular messenger Ca^{2+} activates proteases, lipases and endonucleases. Free radicals are generated and damage the membranes, mitochondria and DNA. This triggers cell death and induces the formation of inflammatory mediators (Woodruff et al, 2011).

Reperfusion injury can occur after the event of ischaemia. Reperfusion injury is the damage to the brain tissue when the blood supply is returned to the ischaemic area after the a period of ischaemia. Several pathophysiology pathway has been suggested as a consequence of the reperfusion injury. Among those are activation of endothelium, excess production of oxygen free radicals, inflammatory responses and leukocyte recruitment, blood brain barrier disruption, increase in cytokine production, and edema formation (Rakesh, Alexander, McKinney, Barbara & Vallabh, 2012).

1.1.2 Current Stroke Treatment and the Future Trend

The current acute stroke treatments (intravenous thrombolysis and endovascular thrombectomy) aim to return blood supply to the ischaemic area. However, they do come with disadvantages. Safety issue on bleeding post-treatment continues to raise concern

among clinicians. Studies show that thrombolysis can result in symptomatic intracranial haemorrhage and major systemic haemorrhage in 6% and 2% of patients respectively (Miller, Simpson, & Silver, 2011). Furthermore, reperfusion may contribute to additional brain injury as the returning blood contains leukocytes that may occlude small vessels and release toxic products (Hallenbeck & Dutka, 1990). The narrow 4.5-hour time window post-stroke for thrombolysis has become one of the major limitations of the use of thrombolysis (Zaheer, Robinson, & Mistri, 2011). Most stroke patients are presented to hospital after the time window as a result of poor knowledge about stroke symptoms and not seeking immediate care (Williams, Bruno, Rouch, Marriott, & MAS, 1997). In most centers, less than 10% of acute ischaemic stroke patient receive intravenous thrombolysis and only an estimated 7-15% of patients could proceed with acute endovascular intervention (Henninger & Fisher, 2016).

Given the very few options on the pharmacotherapy for ischaemic stroke, there is continuous search for novel drugs worldwide. Clinical evidence has suggested that patients with stroke have a slow evolution of brain injury that occurs over a period of hours to days after the insult and this window of opportunity can become a realistic target for therapeutic intervention (Legos & Barone, 2003). Treatment approach could shift from a single acute reperfusion therapy to neuroprotection, with the goal to stop the disease from progressing into a devastating outcome.

Neuroprotective drugs for acute ischaemic stroke to protect the brain from the consequences of ischaemic insult is an active area for drug discovery. They are developed

as a result of our increasing knowledge of the biochemical consequences of an acute ischaemic episode (Green, 2004). However, a large number of clinical trials testing a variety of neuroprotective agents have thus far produced negative results. To date, there is still no neuroprotective agent approved for inhibiting cell injury processes in penumbra following acute vertebral ischaemic insult or reperfusion injury (Hamidon et al., 2012; Savitz, Baron, Yenari, Sanossian, & Fisher, 2017). As a result, besides continuous search of a neuroprotective agent, re-evaluation and improvement on the entire process of drug discovery from *in vitro* screening to clinical trial needs to be made (Holloway & Gavins, 2016). Part of the reasons for failure to identify treatments of stroke are limitations in experimental models and protocol especially in *in vitro* preparations (Ryou & Mallet, 2018). Numerous papers on potential neuroprotective agents have been published but less focus is made on the optimisation on the methods of *in vitro* stroke model. Hence, this project aims to optimise a cell-based *in vitro* stroke model for screening neuroprotective agents.

1.2 Neuroprotective Drug Discovery: *In Vitro* Stroke Model

In vitro models are rapidly developed as a technique to studying molecular and cellular events. In the pipeline of drug discovery, *in vitro* methods allowed screening of hundreds to thousands of compounds per day and enhancing the generation of biological data. Over the past few decades, many researchers have established many *in vitro* and *in vivo* stroke models during exploration on neuroprotective agents. These models help to advance the understanding of the pathophysiology and mechanism of stroke. *In vitro* models of stroke can give a more detailed information that cannot be obtained *in vivo*, especially on the pharmacological properties of new drugs and the effect of the new drugs on neuronal

physiology and biochemistry (Abu-Raya et al., 1999). Although *in vivo* stroke models are more similar to human stroke, large number of animals are needed for statistical power, with its attendant cost and need for care and technical expertise.

It is known that the usage of primary neuron culture is desirable because they are not tumor-derived and hence are more likely to show the properties of neuronal cells *in vivo*. However, primary neuron cultures are difficult to be used and inconvenient to obtain (Chen et al., 2012). Primary cell cultures are not immortal and result in limited number of homogenous cell for experiment. Furthermore, their use in studies on human stroke is extremely limited due to the difficulty in collecting post-mortem tissue at different time points after the onset of stroke (Camós & Mallolas, 2010). Besides, animal tissues are made up of many different types of cell. In order to acquire the cell types of interest, additional procedure to isolate certain type of cells, for example, immunocytochemistry with cell lineage-specific markers, needs to be done (Gordon, Amini, & White, 2013). In addition, mature neuron do not undergone division and there is variability between laboratories (Lahiani, Brand-Yavin, Yavin, & Lazarovici, 2018). Hence, the preparation and culture of primary cells especially for neuronal cells is much more challenging and difficult.

One of the way to overcome the above challenges is to use an established secondary cell line (Gordon et al., 2013). Secondary cell line is an immortal cell line that allows unlimited supply of homogenous cells which are transferable between laboratories. This can reduce the variability between cultures for each experiment. *In vitro* model using secondary

cell line permits a rapid and cost-effective screening for potential compound in the drug discovery pipeline.

In vitro stroke model using secondary cell line has been widely used to screen for potential neuroprotective agents. PC12 cell-based *in vitro* stroke model was one of the commonest cell-based *in vitro* stroke model used in neuroprotective agent screening. (Chen et al., 2012). However, there are very limited papers or reports on the optimisation of the cell culture condition including cell culture media and adhesive substrates on this cell line. In addition, there was increasing reports on the different PC12 variants that has altered response or non-response to nerve growth factor (NGF) to differentiate into neuron-like cells (Hatanaka, 1981; Tischler et al., 2004).

Therefore, this project aimed to optimise several key factors on a PC12 cell-based *in vitro* stroke model with the intention to develop a more relevant *in vitro* stroke model. The optimisation of the *in vitro* model involved three PC12 cell variants, cell culture medium, adhesive substrates and response to NGF-induced differentiation. Many *in vitro* stroke model simulates stroke by removing oxygen and glucose. This is called oxygen glucose deprivation or OGD. The duration of OGD needs to be optimised in the *in vitro* stroke model.

1.3 Tumor-derived PC12 Cell Line

The PC12 cell line (Figure 1.1A) was originally isolated from a rat adrenal medulla tumour (Pheochromocytoma) (Greene & Tischler, 1976). PC12 cells are secondary cell lines that are derived from the tumour and have become immortalized. These have the advantage of being able to be grown fairly easily in cell culture to give unlimited cell numbers as well as minimizing variability between cultures. Treatment of PC12 cells with NGF enables them to be converted from proliferating chromaffin-like cells to non-dividing sympathetic-neuron-like cells with neurites (Greene, 1978) (Figure 1.1B). It was initially used for studying NGF signaling and neuronal differentiation (Tischler, Powers, & Alroy, 2004) and subsequently became the a useful neurosecretion model of exocytosis (Westerink & Ewing, 2008). Differentiation of PC12 cells is reversible when the NGF is absent. Differentiation into neuron-like cells is a useful feature for studies of neuroprotection. It is frequently used for *in vitro* identification of neuroprotectants against a variety of insults including serum deprivation (Greene, 1978), toxic molecules (Gassen, Pergande, & Youdim, 1998; Nakajima et al., 1994) and models of neurodegenerative diseases (Matsumoto, Stojanovic, Holmberg, Kim, & Morimoto, 2005; Walkinshaw & Waters, 1994; Wei et al., 2000).

The PC12 cell line has been used extensively in neuronal research since its establishment in 1976 because of its characteristic of being able to differentiate into neuronal phenotype. It is one of the most frequently employed neuronal cell lines for *in vitro* stroke models in the initial screening for neuroprotective compounds. However, in 1980, the first PC12 clonal variant was isolated using mutagenic approach. It had a flattened morphology

and a loss of response to NGF (Figure 1.2) (Bothwell, Schechter & Vaughn, 1980). Soon after the report, another study also reported on PC12 cell variants that had variety in responsiveness to NGF (Hatanaka, 1981). It has been reported that the signs of spontaneously arising PC12 variants include flat, spiky, rapidly dividing and non-NGF responsive cells (Teng, Angelastro, Cunningham, & Greene, 2006). In 2004, after almost 30 years of establishment of the PC12 cell line, Tischler, Powers and Alroy observed that cells in some laboratories showed loss of NGF responsiveness and other desired traits (Tischler et al., 2004). The change of the phenotype was attributed to the change of culture condition as some of the laboratories switched from using Roswell Park Memorial Institute (RPMI) 1640 media to Dulbecco's modified Eagle's medium (DMEM) high glucose which caused cell flattening because of its higher Ca^{2+} concentration (Tischler et al., 2004). Interestingly, PC12 cells mutagenised to select for clonal variants unresponsive to NGF also resulted in clones that exhibited a flattened epithelial morphology (Bothwell et al, 1980). This was postulated to be due to mutations in structural or regulatory proteins of the cytoskeleton which subsequently affected expression of NGF receptors and neurite extension. Mutagenised NGF-insensitive PC12 variants were reported to change to a flat morphology after 10-30 generations in culture (Bothwell et al, 1980). Besides, another study on PC12 cells response to several neurotoxins also showed that PC12 cell with altered phenotype do not respond uniformly to different stimuli (Mejía, Salgado-Bustamante, Castillo, & Jiménez-Capdeville, 2013). The proposed solution to avoid the loss of desired characteristic of PC12 cell line was to use a new stock of early passage cells and adhere to the original culture conditions (Teng et al, 2006).

The increasing derivations of PC12 cell line since the original cell line which was developed in 1976 by Tischler and Greene (Greene & Tischler, 1976) have resulted in the choice of suitable cells being problematic (Dixon, Loxley, Barron, Cleary, & Phillips, 2005). Dixon and colleagues reported extreme variability between PC12 cells of different laboratories such that it no longer represents a single model. Their comparison of PC12 cells from four different laboratories revealed differences in morphology, adherence, enzyme expression, NGF responsiveness and requirements for media and substratum. Of the four PC12 cell variant tested, there is only one cell variant that was commercially available. However, in recent years, many laboratories that screen for neuroprotectants using an *in vitro* model would use PC12 cells obtained from commercial sources. Therefore, there is a need to compare commercially available PC12 cell variants for their suitability as *in vitro* stroke model and to optimise their cell culture condition (Dixon et al., 2005).

Hence, this study aims to investigate and compare three commonly used commercially-sourced PC12 cell variants on their responses to NGF, suitability as *in vitro* stroke model and the optimisation steps needed to select their best culture conditions. The three PC12 cell variants were obtained from different sources - ATCC (PC 12 Adh), Riken cell bank (PC12 cells) and our collaborator's lab (Neuroscreen-1 cells). According to ATCC, PC12 Adh cells have the morphology of polygonal shape of cells. They are adherent cell type and form cluster. PC12 Adh cells do not synthesis epinephrine. PC12 Adh cell line is an adherent variant of PC12 cell line and has been adapted to Corning CellBIND® flasks to improve cell attachment. They exhibit increased growth rate compared to the traditional PC12 cell line ("PC-12 Adh ATCC® CRL-1721.1™," 2014). As for PC12 cells (Riken), they are originally from Greene and Tischler according to Riken cell bank and possess the properties

of PC12 cell line such as ability to respond to NGF and differentiate, synthesize and store the catecholamine neurotransmitters dopamine and norepinephrine (“Cell Search System-Cell Bank-(Riken BRC)[RCB0009 : PC12],” 2018). The cell morphology is mixture of round and polygonal and has tendency to form cluster. Neuroscreen-1 cell line is a subclone of PC12 cell line. They display several advantages compared to parent PC12 cell line. The cell has 50-80% decreased in doubling time compared to parent PC12 cell line. They can grow as adherent cultures with no tendency toward cell aggregation (“Cellomics® Neuroscreen™-1 Cells,” 2000).

In this study, optimisation of the culture conditions which include cell culture media and cell adhesive substrates were done before comparing three PC12 cell variants on their ability to differentiate. After induced-differentiation with NGF, the cell variant with the highest response to NGF was selected for the subsequent experiments which were optimisation of duration of OGD and validation of the *in vitro* stroke model.

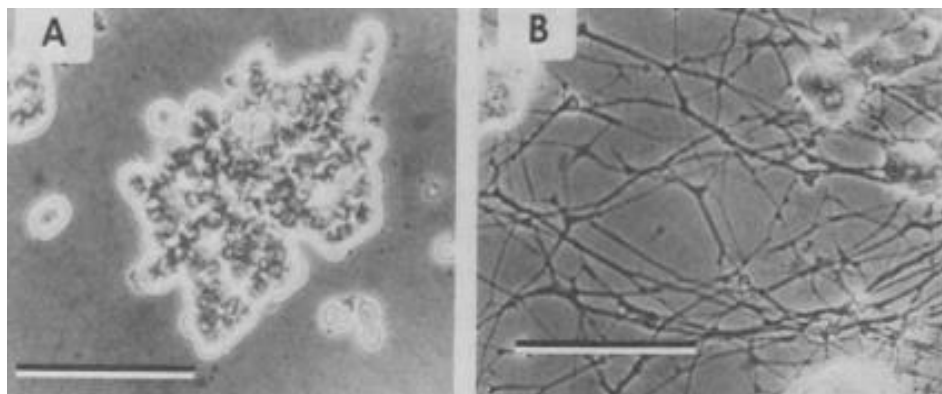


Figure 1.1: Microscopic images of PC12 cells in the absence of NGF (A) and in the presence of NGF for 14 days (B). Bar represents 100 μm . [adapted from Greene and Tischler (1976)].

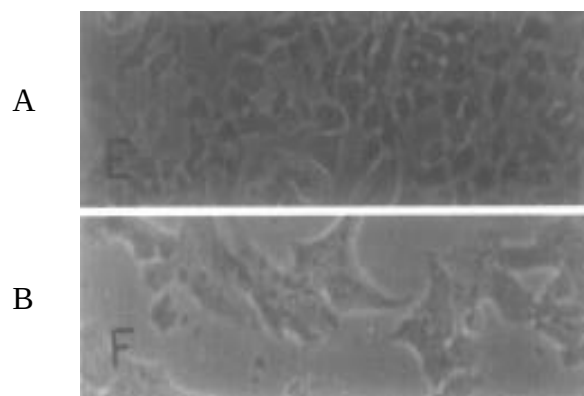


Figure 1.2: Microscopic images of clonal PC12 cell variants with flattened morphology: compact flat (A) and extended flat (B). Bar represent 50 μm . [adapted from Bothwell, Schechter and Vaughn (1980)].

1.4 Cell Culture Media

The two most commonly used culture media for PC12 cells are Roswell Park Memorial Institute medium (RPMI or RPMI 1640) and Dulbecco's Modified Eagle Medium (DMEM) high glucose. Originally, Tischler and Greene used RPMI with defined additives for growing PC12 cells (Greene & Tischler, 1976). However, another group of investigators, who used PC12 cells from the laboratory of the late Gordon Guroff at NIH, used DMEM high glucose for growing PC12 cells (Lelkes, Unsworth, Saporta, Cameron, & Gallo, 2006). Although these two media have been commonly used for PC12 cell culture, so far there is no comparison on genotype and phenotype of PC12 cells in different culture media (Lelkes et al., 2006). Studies on optimization of media for PC12 cells are limited. PC12 cells from different laboratories respond differently to culture medium, NGF and adhesive substrates (Dixon et al., 2005). Selection of a suitable medium for the PC12 cell culture is necessary to ensure a healthy cell culture. Hence, this study aims to compare the two culture media by comparing the morphology of all three PC12 cell variants in the two different media. F12K

medium is recommended by ATCC for PC12 Adh cells but this medium is rarely used for other PC12 cell variants in the cell culture. Therefore, F12K was not used for comparison of the media in this study.

1.5 Serum Free Media

PC12 cells are usually cultured in a medium supplemented with serum (Greene & Tischler, 1976). Serum is an undefined supplement which contains growth factors, hormones and many other unknown factors that cannot be defined easily (Furue et al., 2005). In the screening of compounds or potential neuroprotective agents using an *in vitro* model, avoidance of serum during testing those compounds is desirable. This can help to avoid false positive results caused by those unknown chemicals in the serum. 8-OH-DPAT (5-HT_{1A} agonist), a known neuroprotective agent, was used to validate this system but serotonin (5-HT agonist) is present in serum. To avoid serum effect that contained agonists for the 5-HT_{1A} receptor (e.g. serotonin), a serum-free supplemented medium was necessary during drug treatment on apoptotic cells. In addition, serum free media can enhance the detection of responsiveness of the drug screened. Hence, in this study, serum-free media was used during the validation of the *in vitro* system to avoid the effect of the tested compound which might be presence in the serum.

1.6 Cell Culture Adhesive Substrate

Extracellular matrix (ECM) plays an important role in cell culture by regulating cellular processes including proliferation, adhesion, migration, differentiation and apoptosis (Berrier & Yamada, 2007; Mecham, 2012). Extracellular matrix is made up of approximately 300 proteins and the major components include collagens, proteoglycans, elastin and cell glycoproteins (Yue, 2014). Greene and Tischler grew PC12 cell lines on collagen-coated vessels since the establishment of this cell line in 1976. PC12 cells loosely adhere to normal tissue culture flask in cell culture (Fujii, Massoglia, Savion, & Gospodarowicz, 1982). Besides cell adhesion, extracellular matrix plays a crucial role in neurite outgrowth for PC12 cell differentiation (Lee, Lee, & Kim, 2012). Recently, non-extracellular matrix substratum including poly-D-lysine and poly-L-lysine are also used as an adhesive substrate for PC12 cell culture. Studies showed that different adhesive substrates affected the attachment and differentiation of PC12 cells (Lee et al., 2012; Orłowska et al., 2017). Hence, optimisation of adhesive substrate is necessary in order to choose the most suitable one for the 3 cell variants before proceeding to differentiation studies.

Five commonly-used adhesive substrates, collagen I, collagen IV, poly-D-lysine, poly-L-lysine and laminin for PC12 cell line were selected for this study. All the chosen adhesive substrates have been reported to support the cell growth and differentiation of PC12 cell line (Tomaselli, Damsky & Reichardt, 1987; Susan, Sam, Nailya & Gordon, 2010; Orłowska et al, 2017). Collagen is the most widely used extracellular matrix (ECM) protein for cell culture. Collagen consists of three α -chains which form a triple helix, providing tensile strength to the ECM. Collagen I is the most common fibrillar collagen and is found

in skin, bone, tendons, and other connective tissues. Collagen IV is a primary collagen found in the basement membranes, which underlie epithelial and endothelial cells and surround muscle, fat and nerve cells (Nguyen et al, 2012). Laminin is a glycoprotein of ECM. It is a major component of the basal lamina (one of the layers of the basement membrane), a protein network foundation for most cells and organs. Poly-L-lysine and poly-D-lysine are chemically synthesized ECM useful for promoting cell adhesion to solid substrates. They are positively-charged amino acid polymers. When adsorbed to the culture surface, they increase the number of positively charged sites available for cell binding. As a result, they can enhance electrostatic interaction between negatively charged ions of the cell membrane and the culture surface. They differ in term of stereochemistry and link. In term of their difference in tissue culture, poly-D-lysine is resistant to enzymatic degradation as cells produce proteases that can lyse the poly-L-lysine. This is because poly-L-lysine precursor amino acid occurs naturally but the poly-D-lysine precursor is an artificial product.

1.7 Nerve Growth Factor-induced Differentiation

The PC12 cell line is known to have the properties of differentiating into neuron-like cells. It is a widely used model for studying neuronal biochemistry and neuronal differentiation into sympathetic neurons (Guroff, 1985). NGF induces differentiation in PC12 cell through binding to the tyrosine receptor kinase (TrkA) and results in the activation of intracellular signaling molecules (Kaplan & Miller, 1997). Nerve growth factor binds to TrkA and initiates two major signaling pathways, the MEK/ERK1/2 and PI3K/Akt pathways. Activation of MEK/ERK1/2 and PI3K/Akt cascade promotes neuritogenesis of PC-12 cells. After treatment of NGF, cells cease to proliferate and start to differentiate

(Greene & Tischler, 1976). Induced differentiation needs to be optimised to ensure the cells possess the properties of neuronal cells. Greene first introduced quantification of the response of PC12 cells to NGF in 1977 (Greene, 1977). Based on the neurite outgrowth with the quantification of response to NGF, the response of three variants of PC12 cells can be compared and the cell line with the best response to NGF was selected for use in the *in vitro* stroke model.

1.8 Oxygen-Glucose Deprivation/Reperfusion (OGD/R)

The functions and metabolism of neuronal cells critically depend on sufficient oxygen and glucose supply and the production of ATP from adenosine diphosphate (ADP) (Camandola & Mattson, 2017). Neuronal activity requires a continuous supply of oxygen and glucose, thus, the brain requires more oxygen and glucose than the other organs (Pedersen et al., 1998). When cerebral ischaemia occurs, the blood supply to the brain is interrupted, and brain cells are deprived of the glucose and oxygen they need to function, leading to ischaemic brain injury.

To screen for a neuroprotective agent, a model system which can simulate stroke is needed. OGD/R is widely used as an *in vitro* model for stroke, showing resemblance with the *in vivo* models of an ischaemic brain (Tasca, Dal-Cim, & Cimarosti, 2015). To mimic the stroke-like situation, the studied cell or tissue cultures are incubated under a deoxygenated atmosphere in a glucose-free and serum-free medium. Cells that have undergone optimal duration of OGD show apoptotic changes (Malagelada, Xifró, Miñano, Sabriá, & Rodríguez-Alvarez, 2005). After OGD insult, cells are returned to the normoxia

environment with glucose supply to mimic the condition of “reperfusion” in stroke. During reperfusion, the blood circulation is restored, and oxygen and glucose are resupplied which can result in reperfusion injury. OGD has been used to screen for potential neuroprotective agents in many studies (Chen et al., 2012). Neuroprotection properties demonstrated in such models have been replicated using *in vivo* rat models (Arumugam et al., 2007; Chang et al., 2017).

To ensure the OGD has caused an optimal cell injury, duration of the OGD needs to be optimised. Different durations of exposure of OGD can cause different amounts of cell death. An optimal cell death for investigation of the screening potential neuroprotection should be around 40-60%. Higher cell death values should be avoided as that can cause difficulty in showing the neuroprotection effect (Lahiani et al., 2018). In this study, optimisation of duration of OGD was carried out to select the duration that results in optimal cell apoptosis and cell viability in NGF-differentiated PC12 cells for screening neuroprotectants.

1.9 Apoptosis

Apoptosis or the process of programmed cell death occurs normally. However, if apoptosis occurs in excess or in deficient, it will result in human diseases such as neurodegenerative disease, autoimmune diseases, ischaemic damage and various types of malignancy (Elmore, 2007). Generally, apoptosis can be described by distinct morphology (Kerr, Wyllie, & Currie, 1972) or energy-dependent biochemistry mechanism (Kam & Ferch, 2000).

Cysteine proteases which include caspases are one of the key mediators of apoptosis (Li & Yuan, 2008). In the most cells, caspases are widely present in an inactive form and once activated, the caspases can activate other procaspases and initiate the protease cascade. To date, there are eleven caspases identified and classified into initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11, -12) and effector caspases (caspase-3, -6, -7) (Li & Yuan, 2008). Among the caspases, caspase 3 is identified to be the key mediator for neuronal cell apoptosis (D'Amelio, Cavallucci, & Cecconi, 2010). It was also reported that increased caspase-7 expression correlates with excessive neuronal cell death (Hermel et al, 2004). Hence in this study, caspase 3/7 activities were quantified after induced-apoptosis on differentiated PC12 cells by OGD. Measuring caspase 3/7 activities is relevant to the optimisation of the *in vitro* stroke model. Quantifying the apoptosis after OGD on PC12 cells can indicate the level of apoptotic cell injury.

In the event of stroke, apoptosis occurs in the penumbra area. Develop neuroprotective agent targeting penumbra area to inhibit cell apoptosis is one of the current treatment approaches for stroke. The response of the potential neuroprotective agents can be assessed by measuring the amount of apoptosis reduced. Reduction of apoptosis indicates the rescue of the cells from cell death.

Therefore, OGD of NGF-differentiated PC12 cells permits rapid screening of potential neuroprotective agents. This *in vitro* model is useful as a screening model for neuroprotective agents before validation in primary neuronal culture or *in vivo* systems.

1.10 Validation of the *In Vitro* Stroke Model

This PC12 cell-based *in vitro* stroke model was validated by testing the effect on a known neuroprotective mechanism: activation of 5-Hydroxytryptamine 1A receptors (5-HT_{1A}). Activation of 5HT_{1A} receptor for as neuroprotection of the central nervous system after ischaemic injury is shown in multiple studies (Kukley, Schaper, Becker, Rose, & Krieglstein, 2001; Piera, Beaughard, Michelin, & Massingham, 1995; Ramos et al., 2004). There are many varying hypothesis on the mechanism of activation of 5-HT_{1A} receptor including inhibition of glutamate release (Mauler, Fahrig, Horváth, & Jork, 2001), blockage of voltage sensitive Na⁺ channel (Melena, Chidlow, & Osborne, 2000), activation of NGF signaling pathway (Ahlemeyer, Glaser, Schaper, Semkova, & Krieglstein, 1999) and neuronal hyperpolarization by stimulating postsynaptic 5-HT_{1A} receptors (Khatieb, Fort, Alonso, Jones, & Müllethaler, 1993). Another study showed that one of the 5HT_{1A} agonists, 8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT) can inhibit apoptosis and caspase-3 via the extracellular signal-regulated kinases (ERK1/2) (Figure 1.3) (Adayev, Ray, Sondhi, Sobocki, & Banerjee, 2003).

8-OH-DPAT was used in this study to validate the *in vitro* model by showing reduction of apoptosis compared to control (without 8-OH-DPAT) after OGD-induced cell injury. As a control to 8-OH-DPAT, a 5-HT_{1A} receptor antagonist, WAY 100635 which binds to the same receptors but does not activate the receptors, was used to demonstrate 8-OH-DPAT was working via the 5-HT_{1A} receptor to exert the neuroprotective effect.

Most *in vitro* screening models for neuroprotective agents administer the drugs before or during OGD. However, the vast majority of the stroke cases presented to the hospital for treatment only after the ischaemic event has occurred. Hence, it is important to test whether a neuroprotective agent can reduce neuronal death when applied after the ischaemic insult (Ray et al., 2003). The effect of 8-OH-DPAT was tested in NGF-differentiated cells that have undergone the optimal duration of OGD. Also, during the treatment of 8-OH-DPAT, a serum-free supplemented media was used to restrict using serum that may contain 5HT_{1A} agonist.

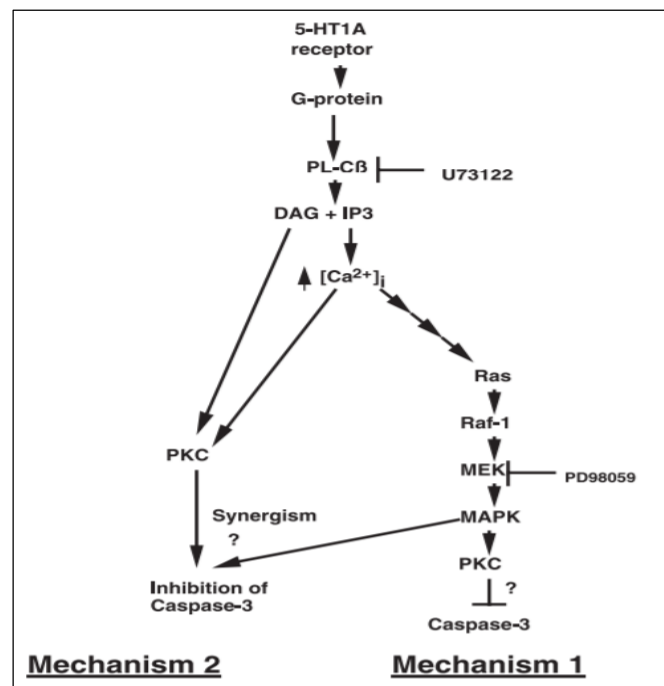


Figure 1.3: Possible mechanisms of 5HT_{1A} receptor-mediated inhibition of caspase-3. In mechanism 2, G protein coupled receptor pathways show that stimulation of phospholipase C_β (PLC_β) by the receptor-activated G protein results in the production of diacylglycerol (DAG) by PLC-catalyzed hydrolysis of phosphoinositide-bis-phosphate. DAG is a potent activator of PKC. It was postulated that protein kinase C (PKC) was activated in a parallel pathway by the ligand bound 5-HT_{1A} receptor. Subsequently, both PKC and mitogen-activated protein kinase (MAPK) could act synergistically to cause inhibition of caspase-3. Mechanism 1 shows that PKC is downstream of the 5-HT_{1A}-R-ERK1/2 pathway and ERK1/2 activation causes stimulation of PKC. Following the stimulation of PKC, caspase-3 was inhibited by the PKC. [adapted from Adayev, Ray, Sondhi, Sobocki & Banerjee, (2003)].

1.11 Hypothesis and Objectives

This study hypothesized that an optimised PC12 cell-based *in vitro* stroke model was effective for screening potential neuroprotective agents.

The general objective of this study was to optimise and set up a PC12 cell-based *in vitro* stroke model for screening potential neuroprotective agents.

The specific objectives of this study were:

- 1.) Optimise the cell culture condition (culture media and adhesive substrates) for three cell variants (PC12 Adh cells, PC12 cells (Riken), NS-1 cells) and adapt them to serum-free supplemented media.
- 2.) Compare the response to NGF of three cell variants to select one cell variant with the best response to NGF.
- 3.) Optimise duration of OGD by cell viability and apoptosis assay.
- 4.) Demonstrate the utility of the model system to screen neuroprotective agents by testing with a known neuroprotectant 8-OH-DPAT which is an agonist to the 5-HT_{1A} receptors.

CHAPTER 2

MATERIAL AND METHODS

2.1 PC12 Cell Culture: PC12 Adh Cells, PC12 Cells (Riken) and Neuroscreen-1 (NS-1) Cells

2.1.1 PC12 Adh Cell Culture

PC12 Adh cells were purchased from the American Type Culture Collection (ATCC CRL-1721.1). The base medium for this cell line was F-12K medium (Gibco, USA) supplemented with 2.5% fetal bovine serum (FBS) (Hyclone, USA) and 15% horse serum (HS) (Gibco, USA). The PC12 Adh cells were maintained at 37 °C in a humidified atmosphere in an incubator with 5% CO₂ (Forma Direct Heat CO₂ Incubator, Model 311, Thermo Scientific, USA). The cells were cultured in a non coated T-25 flask (Nunc, Thermo Scientific, USA) and subcultured every 3-4 days when the cells reached a confluency of 70-80%. During subculturing, culture medium in T-25 was removed and changed to fresh 5.5 mL F-12k medium (pre-warmed to 37 °C). Cells were detached from the flask by scraping with a cell scraper. After scraping, a 10 mL serological pipette was used to triturate the cells for ten times to break up cell clumps. For regular passage, the splitting ratio of 1:7 to 1:6 was used. Around 0.7 mL to 1.1 mL of cells were used for cell passage. Cells carried from 3-13 passages were used for the experiments.

2.1.2 PC12 cells (Riken) Cell Culture

The PC12 cell line was purchased from Riken Cell Bank (catalogue no. RCB0009). PC12 cells (Riken) were maintained at 37 °C in a humidified incubator with 5% CO₂. Cell culture media used was DMEM high glucose (Gibco, USA) with 15% HS and 2.5% FBS. The cells were cultured in a non-coated T-25 flask and subcultured every 3-4 days when the cells reached a confluency of 70-80%. During subculturing, culture medium in T-25 was removed and changed to fresh 5.5 mL DMEM high glucose medium (pre-warmed to 37 °C). Cells were detached from the flask by scraping with a cell scraper. After scraping, a 10 mL serological pipette was used to triturate the cells for ten times to break up cell clumps. For regular passage, the splitting ratio of 1:3 to 1:4.5 was used. Around 1.2 mL to 1.8 mL of cells were used for cell passaging. Cells carried from 3-13 passages were used for the experiments.

2.1.3 Neuroscreen-1 (NS-1) Cell Culture

Neuroscreen-1 cell (NS-1) cell line was a generous gift from Dr Kamila Skieterska, Laboratory of Toxicology, Faculty of Pharmaceutical Sciences, Ghent University. NS-1 cells were cultured in RPMI (Gibco, USA) with 15% HS and 2.5% FBS, Cells were incubated in 5% CO₂ humidified incubator at 37 °C. The cells were cultured in a collagen I-coated T-25 flask and subcultured every 3-4 days when the cells reached a confluency of 70-80%. During subculturing, culture medium in T-25 was removed and changed to fresh 5.5 mL RPMI medium (pre-warmed to 37 °C). Cells were detached from the flask by scraping with a cell scraper. After scraping, a 10 mL serological pipette was used to triturate the cells

for 10 times to break up cell clumps. For regular passage, the splitting ratio of 1:3.5 to 1:5 was used. Around 1 mL to 1.6 mL of cells were used for cell passage. Cells carried from 3-13 passages were used for the experiments.

2.2 Optimisation of Cell Culture Media for Three PC12 Cell Variants

2.2.1 Adaptation of PC12 Cells to Different Media

2.2.1.1 PC12 Adh Cells

PC12 Adh cells were adapted to DMEM high glucose supplemented with 17.5% serum (15% HS, 2.5% FBS) and RPMI supplemented with 17.5% (15% HS, 2.5% FBS), respectively, for comparison in a non-coated T25 flask. PC12 Adh cells were adapted slowly to DMEM high glucose according to adaptation protocol from ATCC[®] Animal Cell Culture Guide ("ATCC Animal Cell Culture Guide," 2014). PC12 Adh cells were subcultured into two T25-flask with the splitting ratio 1:2 to 1:4. One culture was maintained in the original media (F-12K 17.5% serum). The second culture contained half of the new media (DMEM high glucose 17.5% serum) and half of the original media (F-12K 17.5% serum). The original media culture was used as a reference point and a safeguard in case the adapting cells did not survive through the adapting process. The 2 cultures were monitored daily in terms of their confluence rate and morphology. If both the cultures were growing identically, the adapting cells were subcultured into the next passage using the split ratio of 1:2 to 1:4 into 25% of original media and 75% of new media. Subsequently, the adapting cells were

split with 1:2 to 1:4 ratio into 12.5% original and 87.5% new media. Finally, the adapting cells were subcultured into 100% new media with a split ratio of 1:2 to 1:4. If at any point of the adapting process where adapting cells were not performing as well as the reference culture, the adapting cells were cultured in that current media for one to three more passages until they have similar morphology as the cells in original media and relatively comparable growth as the original media culture. The same procedure was repeated for RPMI media with 17.5% serum.

2.2.1.2 PC12 Cells (Riken)

PC12 cells (Riken) were originally cultured in DMEM high glucose 17.5% serum (15% HS, 2.5% FBS) media. Thus, PC12 cells (Riken) were adapted to RPMI 17.5% serum in a non-coated T25 flask. The adaptation process was the same as the process described in Section 2.2.1.1.

2.2.1.3 Neuroscreen-1 (NS-1) Cells

NS-1 cells were originally cultured in RPMI 17.5% (15% HS, 2.5% FBS) media in a collagen I-coated T25 flask. Thus, NS-1 cells were adapted to DMEM high glucose 17.5% serum to compare their morphology. The adaptation process was as described in Section 2.2.1.1.

2.2.2 Comparison of Cell Morphology of PC12 Cells in Different Media

After adaptation to different media, cells were maintained in different media (DMEM high glucose and RPMI for PC12 (Riken) and NS-1 cells) (F-12K, DMEM high glucose or RPMI for PC12 Adh cells) for three passages. At the 3rd passage, cell morphology was compared. The medium which resulted in the best cell morphology compared to other media was selected as the most suitable media for the cell variant. The ‘best’ cell morphology was determined with respect to the cell attachment and neurite outgrowth. The selected media was used for the subsequent experiments.

2.3 Optimisation of Adhesive Substrate of 3 PC12 Cell Variants

2.3.1 Coating Protocol of Collagen I, Collagen IV, Poly-D-lysine, Poly-L-lysine and Laminin

2.3.1.1 Collagen I

Collagen I (3 mg/mL) was purchased from Advanced Biomatrix, USA (catalogue no: 5005). Coating protocol was following the manufacturer’s protocol. Briefly, collagen I was diluted in water or 0.01N HCL to the desired concentration. 90 µg/mL of collagen I was used for coating. An appropriate amount (2 mL for T25, 1 mL for 6-well, 0.5 mL for 12-well and 70 µl for 96-well) of diluted collagen I was added to the culture surface ensuring the entire surface was coated. The culture flask was incubated at room temperature for 1-2 hours. After incubation, remaining material was aspirated. Flask was rinsed with Hank’s balanced

salt solution (HBSS) or phosphate buffered saline (PBS). The coated flask was ready to be used or could be stored at 2-8 °C for 2-3 months.

2.3.1.2 Collagen IV

Collagen IV was purchased from Advanced Biomatrix (catalogue no: 5022). Coating protocol was following the manufacturer's protocol. The lyophilized form of collagen IV (5 mg) was reconstituted with 5 mL of cold, sterile 0.25% acetic acid to a stock concentration of 1 mg/mL. It was mixed for overnight on an orbital shaker at 2-8 °C. Stock collagen IV was diluted to 10 µg/mL working concentration using sterile 0.25% acetic acid. An appropriate amount of the diluted collagen IV was added to the culture surface ensuring the entire surface is coated. The culture flask was incubated at room temperature for 1 to 2 hours. After incubation, the remaining material was aspirated. Flask was rinsed with HBSS or PBS. The coated flask was ready to be used or stored at 2-8 °C for 2-3 months.

2.3.1.3 Poly-D-lysine

Poly-D-lysine solution 1.0 mg/mL was purchased from Merck, USA (catalogue no: A-003-E). Coating protocol was following the manufacturer's protocol. Poly-D-lysine was diluted in sterile water. Fifty µg/mL of poly-D-lysine was used to coat flask. An appropriate amount of diluted poly-D-lysine was added to the culture surface ensuring the entire surface was coated. The culture flask was incubated at room temperature for 3 hours to overnight.

After incubation, the remaining material was aspirated. Flask was rinsed with sterile water or PBS. The coated flask was ready to be used or stored with PBS at 2-8 °C up to 5 days.

2.3.1.4 Poly-L-lysine

Poly-L-lysine solution 0.1 mg/mL was purchased from Merck (USA) (catalogue no: A-005-C). Coating protocol was following the manufacturer's protocol. Poly-L-lysine was diluted in sterile water. Fifty µg/mL of poly-L-lysine was used to coat flask. An appropriate amount of diluted poly-L-lysine was added to the culture surface ensuring the entire surface was coated. The culture flask was incubated at room temperature for 3 hours to overnight. After incubation, the remaining material was aspirated. Flask was rinsed with sterile water or PBS. The coated flask was ready to be used or stored with PBS at 2-8 °C up to 5 days.

2.3.1.5 Laminin

Laminin 1 mg/mL was purchased from Merck (catalogue no: CC095). Coating protocol was following the manufacturer's protocol. Laminin was diluted in PBS to the desired concentration. Ten µg/mL of laminin was used to coat flask. An appropriate amount of diluted laminin was added to the culture surface ensuring the entire surface was coated. The culture flask was incubated at 37 °C for 3 hours. After incubation, the remaining material was aspirated. Flask was rinsed with PBS. The coated flask was ready to be used or stored at 2-8 °C for one week.

2.3.2 Comparison of Cell Morphology of Three PC12 Cell Variants in Different Adhesive Substrates

Three PC12 cell variants were cultured in their respective selected medium in a T25 flask until 70-80% confluence. For PC12 cells (Riken) and NS-1 cells, the selected medium after optimisation of media was DMEM high glucose. As for PC12 Adh cells, the selected medium after optimisation of media was F12K media. Two hundred μ L cells were seeded into 6-well plates which were coated with the different adhesive substrates (as shown in Figure 2.1). Cells were incubated overnight in a 37 °C humidified CO₂ incubator. Cell morphology was assessed after overnight incubation using an Olympus IX71 inverted microscope and images were captured with an Olympus DP72 camera and examined using CellV_F imaging software (Olympus, Japan). Morphology of the cells in the different adhesive substrates was compared and the most suitable adhesive substrate which resulted in the best cell morphology was identified. In cell variants showing no observable differences, the procedure was repeated under serum-free conditions, where the cells were initially cultured in 75% serum-free supplemented media. The cells were then plated on 6-well plates with the different coatings as described above, in 100% serum-free supplemented media. Cellular morphology was observed 24 hours later.

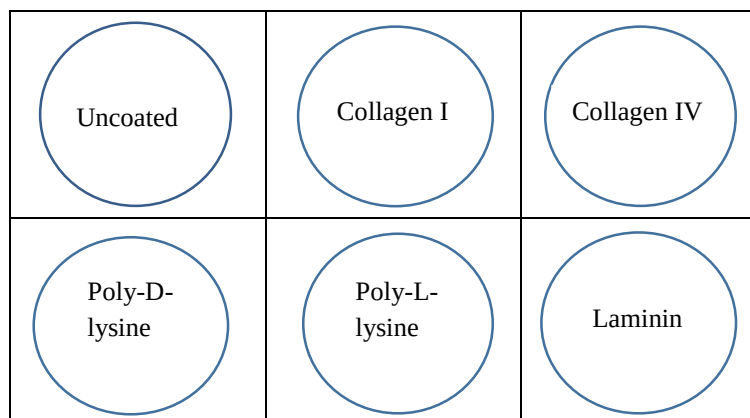


Figure 2.1: Illustration of 6-well plate coated with different adhesive substrate.

2.4 Serum-Free Medium

Serum-free media with supplements was prepared by modification of a published serum-free media for PC12 cells (Ohnuma, Hayashi, Furue, Kaneko, & Asashima, 2006). The serum-free media was prepared according to the formulation as shown below. Six supplements were added into the medium.

2.4.1 Formulation of Serum-free Supplemented Media

The media used to prepare serum-free supplemented media were the selected medium for each PC12 variants after optimisation of culture media. DMEM high glucose was used for PC12 cells (Riken) and NS-1 cells as the result of the optimisation of media showed both of the cell variants preferred DMEM high glucose. F12K medium was used to prepare serum-free supplemented media for PC12 Adh cells as the result of optimisation of

media showed that there was preferential media for PC12 Adh and the recommended media by ATCC was F12K.

Six supplements were added to the selected media for each PC12 cell variants. The 6 supplements were Insulin, Transferrin, Albumin, Selenium, Ethanolamine and β -mercaptoethanol.

2.4.2 Preparation of 6 Supplements

2.4.2.1 Insulin

Insulin from bovine pancreas was purchased from Sigma (USA) (catalogue no: I6634-100 mg). Insulin acts as a growth regulator in the serum-free supplemented medium (Cartwright & Shah, 2002). The concentration of insulin in the serum-free supplemented medium was 30 $\mu\text{g/mL}$. Nine mg of insulin was reconstituted into 3 mL of 4 mM hydrochloric acid to make a 100x stock. Sterile-filter was done. For 100 mL of medium, 1 mL of insulin was added.

2.4.2.2 Apo-transferrin

Apo-transferrin human was purchased from Sigma (catalogue no: T1147-100 mg). Apo-transferrin acts as a transport protein in the serum-free supplemented medium. The

concentration of apo-transferrin in the serum-free supplemented medium was 10 µg/ml. 3 mg of apo-transferrin was reconstituted into 3 mL of PBS to make a 100x stock. Sterile-filter was done. For 100 mL of medium, 1 mL of apo-transferrin was added.

2.4.2.3 Albumin

Bovine serum albumin (BSA) fraction V (7.5%) was purchased from Gibco (USA) (catalogue no: 15260-037-100 mL). BSA acts as a stabilizing protein in the serum-free supplemented medium. The concentration of BSA in the serum-free supplemented medium was 500 µg/mL. For a 100 mL of medium, 670 µL of BSA was added.

2.4.2.4 Ethanolamine

Ethanolamine was purchased from Sigma (catalogue no: E0135-100 mL). Ethanolamine acts as an essential nutrient in the serum-free supplemented medium. The concentration of ethanolamine in the serum-free supplemented medium was 10 µM. Stock concentration was 16.5 M. Ethanolamine was diluted into 33 mM by adding 10 µL of stock into 5 mL of sterile PBS. For a 100 mL of serum-free medium, 30.4 µL of diluted stock (33 mM) was added.

2.4.2.5 β -mercaptoethanol

β -mercaptoethanol was purchased from Sigma (catalogue no: M3148-100 mL). β -mercaptoethanol acts as an essential nutrient in the serum-free supplemented medium. The concentration of β -mercaptoethanol in the serum-free supplemented medium was 10 μ M. Stock concentration was 14.3 M. β -mercaptoethanol was diluted into 28.6 mM by adding 10 μ L of stock into 5 mL of sterile PBS. For a 100 mL of serum-free medium, 35.2 μ L of diluted stock (28.6 mM) was added.

2.4.2.6 Sodium Selenite

Sodium selenite was purchased from Sigma (catalogue no: S9133-1 mg). Sodium selenite acts as a trace element in the serum-free supplemented medium. The concentration of sodium selenite in the serum-free supplemented medium was 10 nM. One mg of sodium selenite was reconstituted into 3 mM. For 100 mL of serum-free medium, 0.34 μ L was added.

2.4.3 Summary of a 100 mL DMEM High Glucose/ F-12K Serum-free Medium with Supplements

Table 2.1 summarizes the content of a 100 mL supplemented DMEM high glucose serum-free media which includes DMEM high glucose or F-12K media, insulin, apo-transferrin, BSA, ethanolamine, β -mercaptoethanol and sodium selenite, with the amount and concentration respectively. DMEM high glucose and F-12K were used as they were the

selected media for PC12 cell Riken, NS-1 cells (DMEM high glucose) and PC12 Adh cells (F12K) after optimisation of media.

Table 2.1: Summary of the content of a 100mL DMEM high glucose serum-free media with supplements.

Supplements / Medium	Amount Added	Target Concentration
DMEM high glucose/F-12K	97 mL	--
Insulin	1 mL	30 µg/mL
Apo-transferrin	1 mL	10 µg/ml
BSA	670 µL	500 µg/ml
Ethanolamine	30.4 µL	10 µM
β-mercaptoethanol	35.2 µL	10 µM
Sodium Selenite	0.34 µL	10 nM

2.4.4 Adaptation to Serum-free Supplemented Media

2.4.4.1 PC12 Adh Cells

Adaptation of PC12 Adh cells from F-12K with serum to F-12K without serum was carried out according to media adaptation protocol in the ATCC Animal cell Culture Guide ("ATCC Animal Cell Culture Guide," 2014). PC12 Adh cells were subcultured into two T25-flask with the splitting ratio 1:2 to 1:4. One culture was maintained in the original media (F-12K 17.5% serum). The second culture contained half of the new media (F-12K no serum with supplements) and half of the original media (F-12K 17.5% serum). The original media culture was used as a reference point and a safeguard in case the adapting cells did not survive through the adapting process. The two cultures were monitored daily in terms of their confluence rate and morphology. If both the cultures were growing identically, the adapting cells were subcultured into the next passage using the split ratio of 1:2 to 1:4 into 25% of original media and 75% of new media. Subsequently, the adapting cells were split

with 1:2 to 1:4 ratio into 12.5% original and 87.5% new media. Finally, the adapting cells were subcultured into 100% new media with a split ratio of 1:2 to 1:4. If at any point of the adapting process where adapting cells were not performing as well as reference culture, the adapting cells were cultured in that current media for one to three more passages until they have similar morphology as the cells in original media and relatively comparable growth as the original media culture.

2.4.4.2 PC12 Cells (Riken)

PC12 cells (Riken) were originally cultured in DMEM high glucose 17.5% serum. Adaptation of PC12 cells (Riken) from DMEM high glucose 17.5% serum to DMEM high glucose no serum with supplements were carried out according to media adaptation protocol in Section 2.4.4.1.

2.4.4.3 Neuroscreen-1 (NS-1) Cells

NS-1 cells were cultured in DMEM high glucose 17.5% serum. Adaptation to serum-free supplemented media was carried out as described in Section 2.4.4.1.

2.5 Nerve Growth Factor (NGF)-induced Differentiation of Three PC12 Cell Variants

2.5.1 Nerve Growth Factor (NGF) Reconstitution and Storage

NGF-7S 0.1 mg from mouse submaxillary glands was purchased from Sigma (catalogue no: N0513). NGF was reconstituted in sterile PBS containing 0.5% BSA. Four hundred μL of PBS containing 0.5% BSA was used to dilute the NGF to produce 0.25 $\mu\text{g}/\mu\text{L}$ stock concentration. NGF stock solution was aliquoted into 5 μL per tube and stored at $-80\text{ }^{\circ}\text{C}$. On the day of NGF treatment, 120 μL of medium were added into the NGF tube and final concentration was 10 $\mu\text{g}/\text{mL}$.

2.5.2 PC12 Adh Cell Differentiation

PC12 Adh cells were cultured in F-12K medium with 17.5% serum. (15% HS, 2.5% FBS). Cells with confluency 70%-80% were scraped and triturated. Total cell number was counted with a haemocytometer and seeded into 6-well plates coated with poly-D-lysine (ThermoFisher Scientific, catalogue no: 152035) with seeding density 3×10^4 cells per well in F-12K 17.5% serum. Cells were incubated at $37\text{ }^{\circ}\text{C}$ in a 5% CO_2 -humidified incubator overnight. On the next day, the medium was changed to F-12K 1% HS and different concentrations of NGF ranging from 0 ng/mL to 300 ng/mL (w/v) were added to investigate the optimum concentration for neurite outgrowth activity. Low serum medium (1-2%) was used for NGF-differentiation as low serum medium reprogrammes cells from proliferation state to differentiation state. 1% HS was used for PC12 Adh cells whereas 2% HS was used for NS-1 cells and PC12 cells (Riken) after optimisation was done (data not shown). Neurite

outgrowth activity was observed daily until day 4. Images were taken for semi-quantitative assessment (Nicholas M Radio & Mundy, 2008) as described in Section 2.5.5.

2.5.3 PC12 Cells (Riken) Cell Differentiation

PC12 cells (Riken) were cultured in DMEM high glucose with 17.5% serum (15% HS, 2.5% FBS). Cells with confluency 70%-80% were used for the experiment. Cells were scraped and triturated. Total cell number was counted with a haemocytometer and seeded into a 12-well plate (coated with collagen IV 10µg/mL) with seeding density 15×10^3 cells per well in DMEM high glucose 17.5% serum. Cells were incubated at 37 °C in a 5% CO₂-humidified incubator overnight. On the next day, the medium was changed to DMEM high glucose 2% HS and different concentrations of NGF ranging from 0 ng/mL to 300 ng/mL (w/v) were added to investigate the optimum concentration for neurite outgrowth activity. Neurite outgrowth activity was observed daily until day 4. Images were taken for semi-quantitative assessment as described in the Section 2.5.5.

2.5.4 Neuroscreen-1 (NS-1) Cell Differentiation

NS-1 cells were cultured in DMEM high glucose with 17.5% serum (15% HS, 2.5% FBS). Cells with confluency 70%-80% were used for the experiment. Cells were scraped and triturated. Total cell number was counted with a haemocytometer and seeded into a 12-well plate (coated with collagen IV 10µg/mL) with seeding density 15×10^3 cells per well in DMEM high glucose 17.5% serum. Cells were incubated at 37 °C in a 5% CO₂-humidified

incubator overnight. On the next day, the medium was changed to DMEM high glucose 2% HS and different concentration of NGF ranging from 0 ng/mL to 300 ng/mL (w/v) were added to investigate the optimum concentration for neurite outgrowth activity. Neurite outgrowth activity was observed daily until day 4. Images were taken for semi-quantitative assessment as described in the Section 2.5.5.

2.5.5 Neurite Scoring

A cell was scored positive as a neurite-bearing cell when the neurite length was one time or more than the cell body diameter. Total number of cells counted was at least 150 cells taken from at least four randomly chosen fields per well (Smalheiser & Schwartz, 1987). Cells were examined using an Olympus IX71 inverted microscope with a 20x objective (NS-1 cells and PC12 cells (Riken)) and 10x objective (PC12 Adh cells). Images were captured with Olympus DP72 Camera and examined using Cell^VF imaging software (Olympus, Japan). The percentage of neurite-bearing cells was quantified by the proportion of neurite-bearing cells over the total number of cells.

$$\text{Percentage of neurite-bearing cells} = \frac{\text{Total number of neurite-bearing cells}}{\text{Total number of cells}} \times 100\%$$

2.5.6 Comparison of Percentage of Neurite-bearing Cells

Scoring of total cell number and total neurite-bearing cell number were done with Image J software (NIH, USA). Data collected was documented in Microsoft Excel. Data analysis was done using Microsoft Excel to obtain the percentage of neurite-bearing cells. The percentage of neurite-bearing cells for all 3 cell variants were compared after 4 days of differentiation. All the experiments were repeated 3 times. Statistical analysis was done using GraphPad Prism 5 software, USA.

2.6 Oxygen Glucose Deprivation (OGD)

2.6.1 Removal of Glucose

On the day of experiment for OGD, the normal DMEM high glucose culture medium (selected media for NS-1 cells) was replaced with DMEM without glucose and serum (Gibco, USA) to remove glucose from the cells.

2.6.2 Removal of Oxygen

After the medium was replaced with the glucose-free and serum-free medium, 96-well plates with cells were put into an anaerobic environment by using BD BioBag[®] environmental chamber (Type A Multiplate) from Becton Dickson, USA (catalogue no:

M773125(01)) following manufacturer's instructions (Tatyana Adayev, El-Sherif, Barua, Penington, & Banerjee, 1999).

Briefly, in each sealed Bio-Bag (Type A) Multi-Plate system, there was a generator consisting of an ampule of a weak hydrochloric acid solution and two gas-generating tablets. When the ampule was crushed, the acid reacted with the tablets, resulting in the formation of a mixture of gases including hydrogen and carbon dioxide. Hydrogen, in the presence of the palladium catalyst, quickly reacted with the atmospheric oxygen in the bag to form water. Removal of oxygen was indicated by the change in colour (from pink to colourless, of the resazurin indicator included with the system).

2.6.3 Test Procedure of BD-BioBag®

One catalyst cup was placed in the bottom of the bag. 96-well plate was placed in the Bio-Bag (Type A) Multi-Plate, right side up. Gas generator/anaerobic indicator was placed in the bag perpendicular to the plates and at the open end of the bag. The tablets should be closer to the sealed end of the bag. Second catalyst cup was placed in the bag at the open end of the bag. The bag was heat-sealed carefully twice 2 cm apart so that it was airtight. The indicator ampule was crushed. The solution from the crushed ampule would saturate the pledget beside the indicator. A pink colour indicating transient aerobic conditions would develop in the indicator in approximately 1 min. The generator ampule was crushed. The dilute hydrochloric acid would be observed flowing from the generator ampule to the two gas generator tablets; gas would be seen to evolve from the tablets. The sealed end was

elevated slightly (approximately 1 inch.) to allow liquid to flow into the tablet chamber. The bag must remain in a flat position for about 60 seconds until all gas has evolved. BD-BioBags which contained a 96-well plate in each bag were put into a 37 °C 5% CO₂-humidified incubator for indicated durations (0, 1, 2, 3, 4.5 and 6 hours).

2.7 Optimisation of Duration of Oxygen Glucose Deprivation (OGD) using Cell Viability Assay (MTT Assay)

Cell viability assay which was used to assess cell survival in this project was MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay.

2.7.1 MTT Reagent Preparation

Thiazolyl Blue Tetrazolium powder was purchased from Sigma (catalogue no: M5655-1G). MTT powder 30 mg was diluted with 6 mL of 1X PBS (no Ca⁺, no Mg⁺) to a concentration of 5 mg/mL (10X stock). The working concentration for MTT assay is 0.2 mg/mL to 0.5 mg/mL. After mixing the powder in 1X PBS and vortexing vigorously, the solution was sterile filtered in laminar flow cabinet. MTT reagent was ready to use or stored at -20 °C, protected from light.

2.7.2 Procedure of Optimisation of Oxygen Glucose Deprivation (OGD) using MTT Assay

2.7.2.1 96-well Plate Coating

Cellbind[®] tissue culture clear flat bottom 96-well plates were purchased from Corning, USA (catalogue no: C62-3300). Collagen IV was used to coat the plate. Collagen IV was used because it was the best suitable adhesive substrate for NS-1 cell after optimization. Acetic acid 0.25% was used to dilute Collagen IV to the final concentration of 10 µg/mL. Seventy µL of Collagen IV (10 µg/mL) was added into each well. 96-well plates were incubated for 1-2 hours. Plates were then rinsed with HBSS. 96-well plates were ready to be used or stored at 4 °C.

2.7.2.2 Cell Seeding

NS-1 cell line were selected for optimization of duration of OGD as it was the cell variant with best response to NGF. NS-1 cells at a confluency of 70% in a T-25 flask were scraped and triturated with 5.5 mL DMEM high glucose 17.5% serum medium. Cells were then seeded into 96-well plates (coated with collagen IV 10 µg/mL) at a density of 4×10^3 cells per well in 100 µL DMEM high glucose 17.5% serum. Cell density was decided after optimisation done (refer to Appendix C). Cell seeding for each sample was done in triplicate wells. Cells were incubated overnight at 37 °C in a 5% CO₂-humidified incubator.

2.7.2.3 Induction of Cell Differentiation with Nerve Growth Factor (NGF)

After overnight incubation, cells were examined with an inverted microscope. NGF differentiation was carried out by changing culture medium to DMEM high glucose 2% HS containing 150 ng/mL of NGF (differentiating medium). Each well contained 100 μ L of differentiating medium. Differentiating medium was changed every 2 days. After 3 days, cells were examined using an inverted microscope to ensure the cells were differentiated.

2.7.2.4 Induction of Cell Injury by Oxygen Glucose Deprivation (OGD)

Cells were subjected to OGD as described in the Section 2.6. OGD was carried out by using different durations which ranged from 0, 1, 2, 3, 4.5 to 6 hours to determine the optimal duration of OGD. The durations of 0, 1, 2, 3, 4.5 and 6 hours were selected after a preliminary study on the optimisation of the durations ranging from 0 hour to 24 hours of OGD (data not shown) was done.

2.7.2.5 Measurement of Cell Viability using MTT assay

After undergoing OGD, 96-well plates were centrifuged at 1000 x g for 3 minutes at room temperature (Centrifuge 5810R, Eppendorf, Germany). The medium was changed to medium containing 0.5 mg/mL of MTT (medium containing glucose). Cells were incubated at 37 °C in a 5% CO₂-humidified incubator for 3 hours. Centrifugation for 3 minutes at 1000 x g was carried out. The supernatant was carefully discarded. One hundred μ L of

DMSO was then added into each well to dissolve the MTT formazan crystal. Blank wells consisted of 100 µL of DMSO without cells. 96-well plates were covered with aluminium foil and put on an orbital shaker to mix for 10-15 minutes. The extent of the reduction of MTT was determined by measuring the absorbance at 590 nm using a microplate reader (Infinite 200 Pro, Tecan, Switzerland).

2.8 Optimisation of Duration of Oxygen Glucose Deprivation (OGD) using Apoptosis Assay (Caspase 3/7 Assay)

After stroke, apoptosis is a major cause of the death of brain neurons. Hence, the optimal duration of OGD was also tested using apoptosis as a readout. The cell apoptosis assay which was used to assess cell apoptosis in this project measured caspase-3 and caspase-7 activity. Caspase-3 and caspase-7 are the effector caspases that are released during early cell apoptosis. The caspase-3 and caspase-7 activities were measured by using Caspase-Glo[®] 3/7 Assay Kit (Promega, USA, catalogue no: G8090).

2.8.1 Procedure of Optimisation of Oxygen Glucose Deprivation (OGD) using Caspase 3/7 Assay

The procedure of 96-plate coating, cell seeding, induction of cell differentiation by NGF and induction of cell injury by OGD were carried out as described in Section 2.7.2.1 to Section 2.7.2.4. After OGD, caspase 3/7 assay was carried out as described in the following sections.

2.8.1.1 Measurement of Apoptosis using Caspase 3/7 Assay

Apoptosis was assayed by using the detection of caspase-3 and caspase-7 activities after cell injury. Caspase-Glo® 3/7 assay kit was used. This assay provides a proluminescent caspase-3/7 DEVD-aminoluciferin substrate and a proprietary thermostable luciferase in a reagent optimized for caspase-3/7 activity, luciferase activity and cell lysis. Adding the single Caspase-Glo® 3/7 Reagent results in cell lysis, followed by caspase cleavage of the substrate. This liberates free aminoluciferin, which is consumed by the luciferase, generating a "glow-type" luminescent signal that is proportional to caspase-3/7 activity.

Caspase-Glo® 3/7 Assay was carried out according to the manufacturer's instruction. Briefly, Caspase-Glo® 3/7 Buffer and lyophilized Caspase-Glo® 3/7 Substrate were equilibrated to room temperature before use. The contents of the Caspase-Glo® 3/7 Buffer bottle was transferred into the Caspase-Glo® 3/7 Substrate bottle. The substrate was mixed by swirling thoroughly until the substrate was dissolved to form the Caspase-Glo® 3/7 Reagent.

After different durations of OGD, 96-well plates were removed from the incubator and allowed to equilibrate to room temperature. Hundred µL of reagent was added to the cells. Blank wells consisted of 100 µL of glucose-free and serum-free DMEM and 100 µL reagent. Contents of the 96-well plates were gently mixed at 400 rpm for 30 seconds and incubate at 25 °C in an incubator for 30 minutes. After incubation, the luminescence was measured using a microplate counter (Microbeta LumiJet, Perkin Elmer, USA).

For non-OGD plate, medium was changed to 100 μ L of glucose-free and serum-free DMEM. 100 μ L of reagent was added to each well containing cells. Blank wells consisted of 100 μ L of glucose-free and serum-free DMEM and 100 μ L reagent without cells. Contents of the 96-well plates were gently mixed at 400 rpm for 30 seconds and incubated at 25 °C in an incubator for 30 minutes. After incubation, the luminescence was measured using a microplate counter (Microbeta LumiJet, Perkin Elmer, USA).

2.9 Validation of the *In Vitro* Stroke Model by Testing the Neuroprotective Effect of 8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT)

This *in vitro* stroke model was validated by testing with a reported neuroprotective agent, 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist that activates 5-HT_{1A} receptors. 8-OH-DPAT was purchased from Tocris, USA (catalogue no: 0529). As a negative control to 8-OH-DPAT, WAY 100635 maleate (Tocris, USA, catalogue no: 4380) which is an antagonist to the 5HT_{1A} receptors was used. The in-vitro stroke model was validated by showing reduction of cell apoptosis by 8-OH-DPAT.

2.9.1 Procedure of Validation *In Vitro* Stroke Model

The procedure of 96-plate coating, cell seeding, induction of cell differentiation by NGF were carried out as described in Section 2.7.2.1 to Section 2.7.2.3 except that the cell seeding density was 2.5×10^3 cells per well. Cell density was decided after optimisation done (refer to Appendix C). 3 hours of OGD was selected as the optimal duration for the *in vitro* stroke model after optimisation. After NGF differentiation, induction of cell injury by

OGD with 3 hours of OGD was carried out. 3 hours was selected as the optimal duration of OGD for inducing cell injury in NS-1 cells. OGD was carried out as described in Section 2.6.

2.9.1.1 Reperfusion in Normal Culture Condition

After 3 hours of OGD-induced cell injury, the 96-well plate was removed from the anaerobic chamber. Media with glucose and 6 supplements were added into each well to replace the requirement of serum for normal cell culture. The target of each supplement was described in Section 2.4.3.

2.9.1.2 Treatment with 8-OH-DPAT and WAY 100635

In the OGD-treated 96-well plate, cells were treated with 1 μ M of 8-OH-DPAT per well (triplicate). As a control to 8-OH-DPAT, 1 μ M of WAY 100635 maleate was added to the OGD treated 96-well plates cells in another 3 wells.

Control wells had no drug treatment. After treatment with the drugs, OGD treated 96-well plates were returned to 37 °C in a 5% CO₂-humidified incubator for 24 hours. Non-OGD treated 96-well plate was maintained in 37 °C in a 5% CO₂-humidified incubator for 24 hours as a control to OGD-treated cells.

2.9.1.3 Measurement of Apoptosis using Caspase-3/7 Assay

After 24 hours, apoptosis assay (caspase 3/7 assay) was carried out on the cells in both OGD-treated and non-OGD treated 96-well plates according to the manufacturer's instruction as described in Section 2.8.1.1.

2.10 Statistical Analysis

All data was analyzed using GraphPad Prism 5 software (NIH, USA). The results were presented as the mean $\pm$ SD. Data sets were tested for statistical significance using unpaired t-test for comparing 2 groups of data or one-way analysis of variance (ANOVA) followed by post hoc Tukey's comparison test for comparing 3 or more groups of data. A *P*-value less than 0.05 was considered statistically significant.

2.11 Summary of Materials and Methods

The summary of methods in setting up a PC12 cell-based *in vitro* model for screening potential neuroprotective agents is illustrated in Figure 2.2.

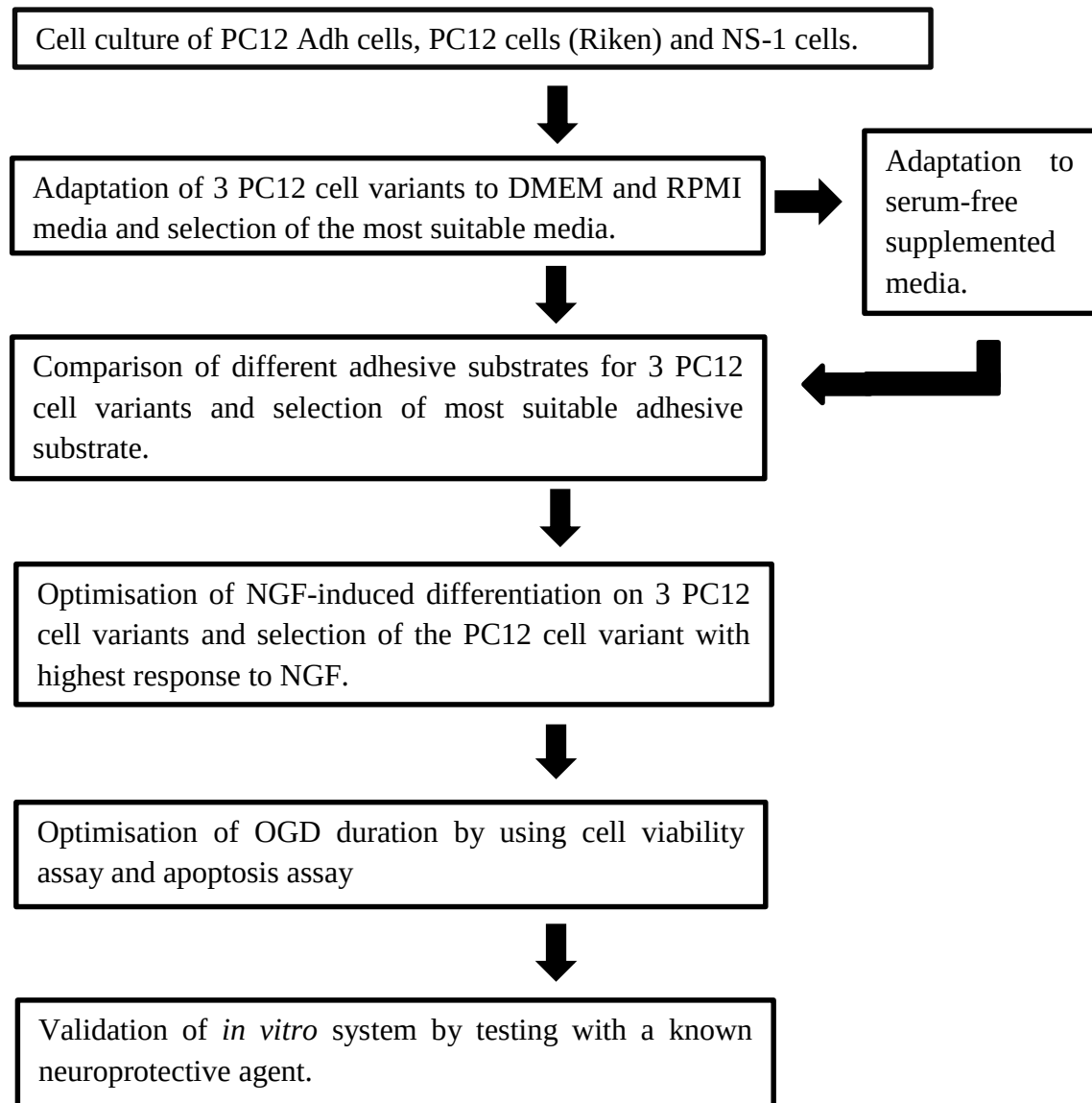


Figure 2.2: Workflow diagram of methods in setting up a PC12 cell-based *in vitro* stroke model

CHAPTER 3

RESULT

3.1 Optimisation of Cell Culture Media of Three PC12 Variants by Comparing Cell Morphology

3.1.1 PC12 Adh Cells

ATCC recommends F12K medium for PC12 Adh cells. PC12 Adh cells were successfully adapted from F-12K supplemented with 17.5% serum (15% HS, 2.5% FBS) to DMEM high glucose supplemented with 17.5% serum (15% HS, 2.5% FBS) and RPMI supplemented with 17.5% serum (15% HS, 2.5% FBS). Figure 3.1 shows the cells were able to grow and had similar morphology in these three media. Cell morphology was normal (a mixture of polygonal and longish shape) and cell growth was relatively comparable in all three media. F-12K medium was selected as the medium for the subsequent experiments as it was recommended by ATCC. The experiment was repeated three times.

3.1.2 PC12 Cells (Riken)

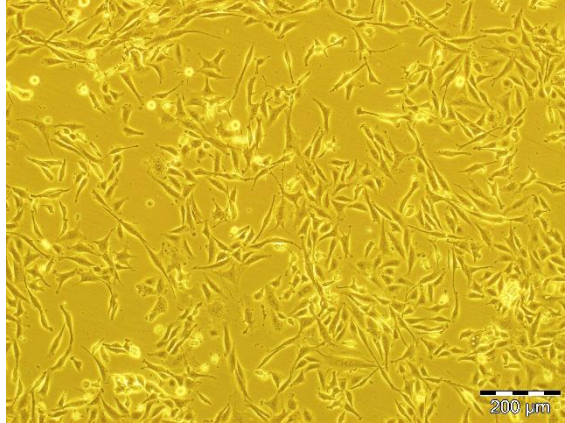
Riken cell bank recommends DMEM for PC12 cells while RPMI was recommended for PC12 cells originally (Greene & Tischler, 1976). PC12 cells (Riken) were adapted to RPMI supplemented with 17.5% serum (15% HS, 2.5% FBS) from DMEM high glucose supplemented with 17.5% serum (15% HS, 2.5% FBS) but the cells were unable to adhere to the flask when adapted to RPMI (17.5% serum) media (Figure 3.2B). The cells in RPMI

medium were rounded up and floating. In DMEM high glucose (Figure 3.2A), the cells were healthy with good morphology. Hence, comparing DMEM high glucose vs RPMI, the best suitable medium for PC12 cells (Riken) was DMEM high glucose 17.5%. The experiment was repeated three times

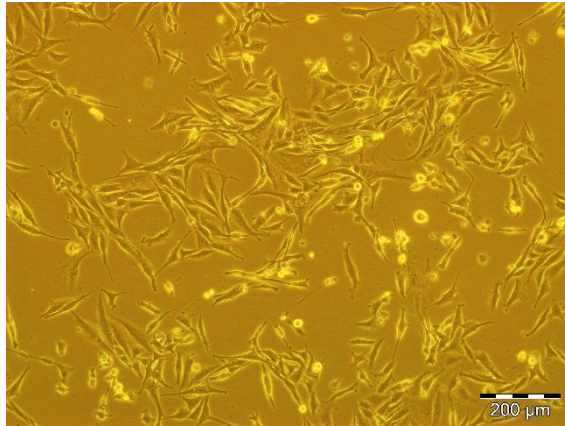
3.1.3 Neuroscreen-1 (NS-1) Cells

NS-1 cells was originally supplied by Cellomics[®] with RPMI as the recommended medium. NS-1 cells were successfully adapted to DMEM high glucose supplemented with 17.5% serum (15% HS, 2.5% FBS) from RPMI supplemented with 17.5% serum (15% HS, 2.5% FBS). More NS-1 cells in DMEM high glucose medium (Figure 3.3A) had neurite extension compared to RPMI. More floaters and debris were found using RPMI (Figure 3.3B). Hence DMEM high glucose is a better medium for NS-1 cells than RPMI. The experiment was repeated three times

A



B



C

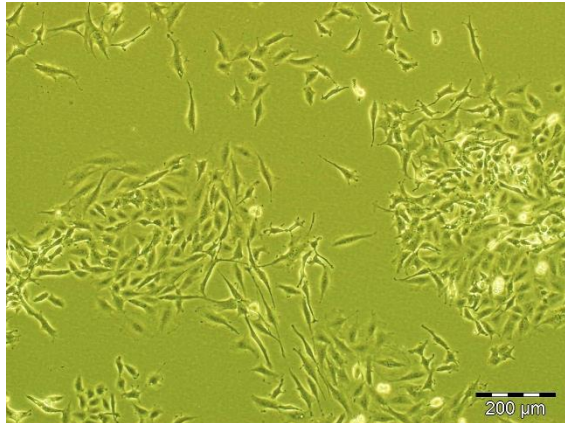
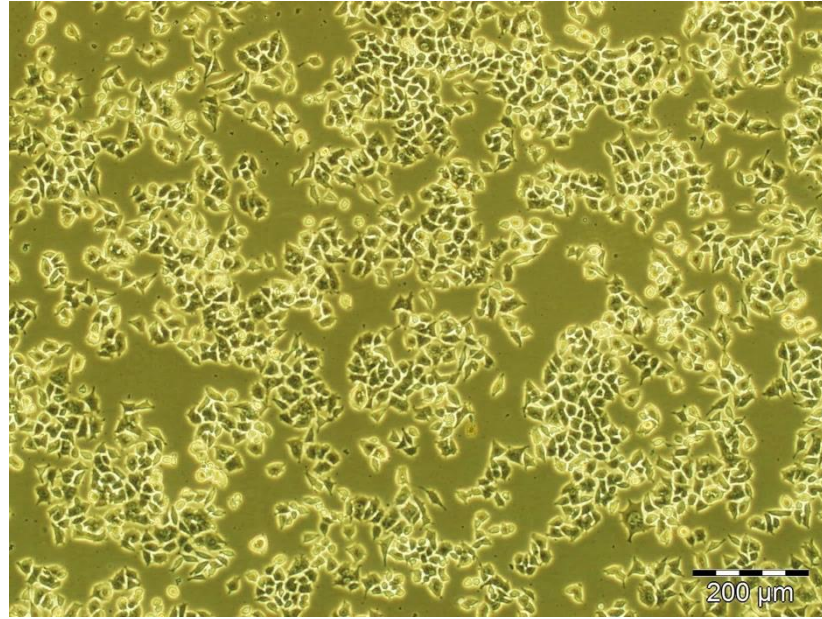


Figure 3.1: Microscopic images of PC12 Adh cells at 100x magnification in different media. Cells were cultured in F-12K media with 17.5% serum (A), DMEM high glucose media with 17.5% serum (B) and RPMI media with 17.5% serum (C), respectively. Cells morphology was a mixture of polygonal and longish in all three media.

A.



B.

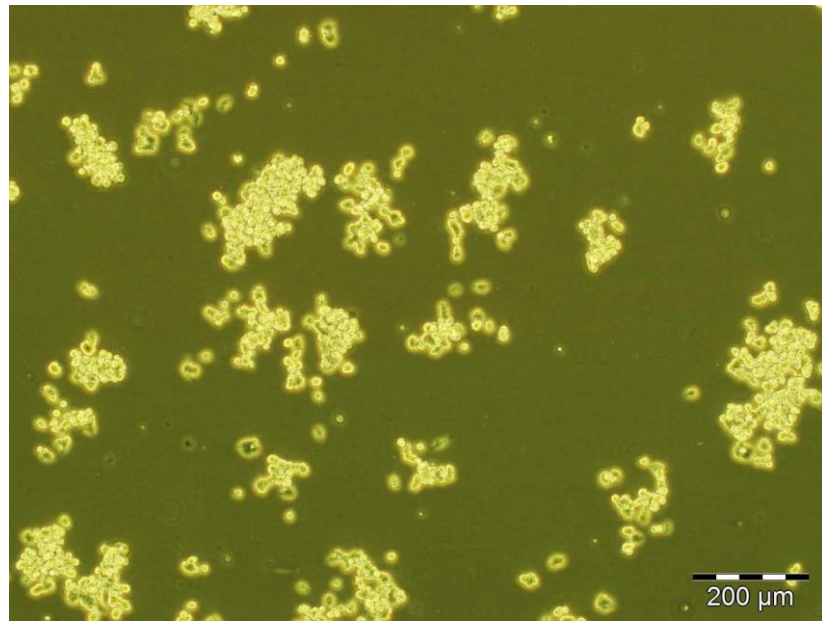


Figure 3.2: Microscopic images of PC12 cells (Riken) at 100x magnification in different media. Cells cultured in DMEM high glucose medium with 17.5% serum showed normal morphology (A). Cells cultured in RPMI medium with 17.5% serum showed cells were unable to adhere, rounded and formed clumps (B).

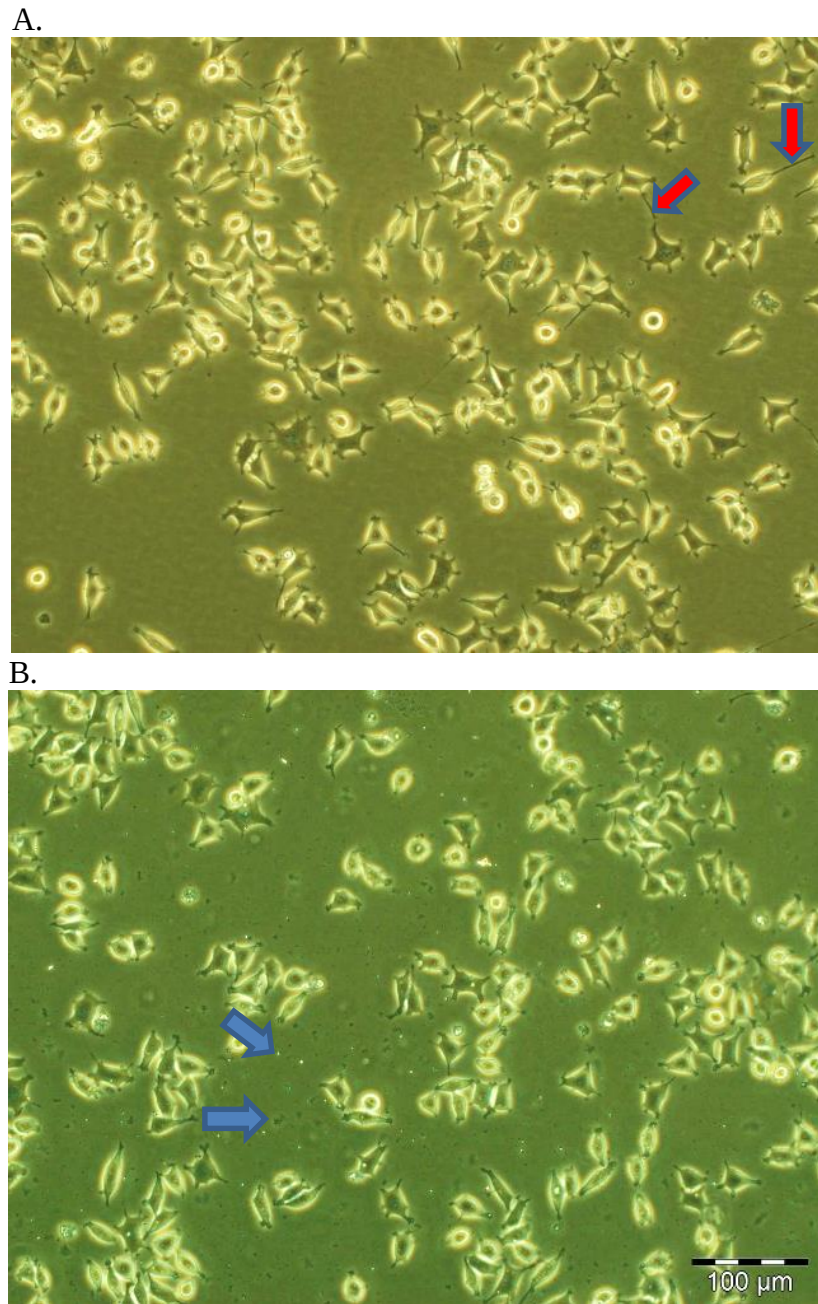


Figure 3.3: Microscopic images of NS-1 cells at 200x magnification in different media. Cells in DMEM high glucose medium with 17.5% serum adhered well with extension of neurites (indicated by arrows) (A). Cells cultured in RPMI medium with 17.5% serum showed normal morphology but with more cell debris and floaters noted (indicated by arrows) (B).

3.2 Optimisation of Cell Adhesive Substrate for Three PC12 Variants by Comparing Cell Morphology

3.2.1 Neuroscreen-1 (NS-1) Cells

Cellomics® recommends NS-1 cells to be cultured in collagen-I coated vessels. Morphology of NS-1 cells in different adhesive substrates was compared. Results showed that collagen IV was the best adhesive substrate for NS-1 cells in full serum (Figure 3.4). Cells in collagen IV-coated flask were flatter and had better spread compared. In non-coated and other coated flasks, cells were smaller with less spread and brighter. The experiment was repeated three times.

3.2.2 PC12 Cells (Riken)

Riken cell bank does not recommend any adhesive substrate in normal culture of PC12 (Riken) cells (in full serum). Morphology of PC12 cells in different adhesive substrates was compared (in full serum DMEM high glucose). In DMEM high glucose 17.5% serum, all types of substrates gave better morphology than the non-coated flask (Figure 3.5). Cells had better spread in the coated flasks. However, there was no difference in morphology between different adhesive substrates (Figure 3.5 B-F). Hence, comparison of different adhesive substrates was further investigated by using serum-free supplemented media. One of the major functions of serum in culture media is to provide attachment and spreading factors to facilitate cell attachment to the substratum (Brunner, Frank, Appl, Schoffl, Pfaller & Gstraunthaler, 2010). Hence, cells cultured in serum-free media may

require an adhesion substrate to remain attached. It follows that culture of PC12 cells in serum-free media may reveal its preferential adhesion substrate. Therefore, we tested the culture of PC12 Riken and PC12 Adh cells in serum-free supplemented media. In serum-free supplemented DMEM high glucose, collagen IV-coated flask was the best adhesive substrate for PC12 cell (Riken) as cells were more spread out and darker as seen by phase contrast microscopy (Figure 3.6C). In serum-free supplemented DMEM high glucose in collagen I-coated flask, Poly-D-lysine-coated flask, Poly-L-lysine-coated and Laminin-coated flask, the majority of the cells were rounded, smaller and brighter as seen by phase contrast microscopy (Figure 3.6B, D, E, F). In the non-coated flask, all cells were rounded, small and not adhered to the flask (Figure 3.6A). This observation suggested that the cells were more firmly adhered in collagen IV-coated flask in serum-free supplemented DMEM high glucose. Hence, collagen IV was selected as the most suitable adhesive substrate for PC12 cells (Riken). The experiment was repeated three times

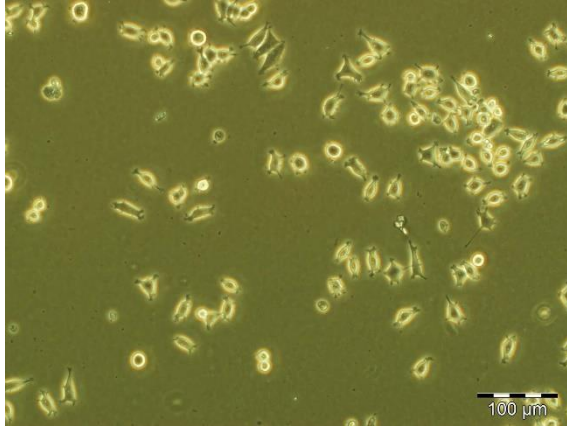
3.2.3 PC12 Adh Cells

ATCC does not recommend any adhesive substrate in the culture of PC12 Adh cells. Cells were cultured in F-12K medium supplemented with 17.5% serum in different adhesive substrates (collagen I, collagen IV, poly-D-lysine, poly-L-lysine and Laminin) in 6-well plates. However, no differences in cell morphology was seen (Figure 3.7). To help in differentiating the different substrate, cells were cultured in F-12K serum-free supplemented medium with these different adhesive substrates in 6-well plates. However, all the cells were unable to adhere and grow in serum-free supplemented F-12K medium (Figure 3.8). Hence,

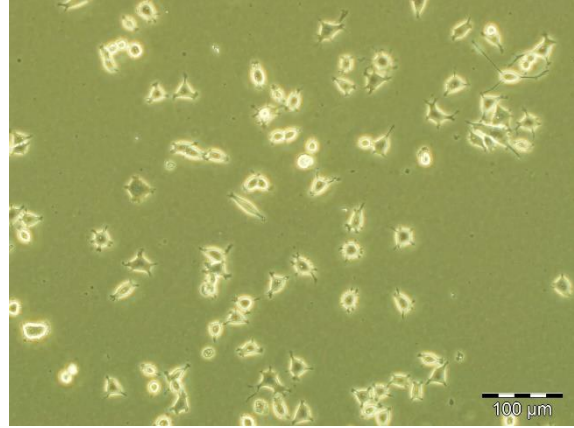
adhesive substrate did not appear necessary in the normal culture of PC12 Adh cells. The experiment was repeated three times.

Full serum

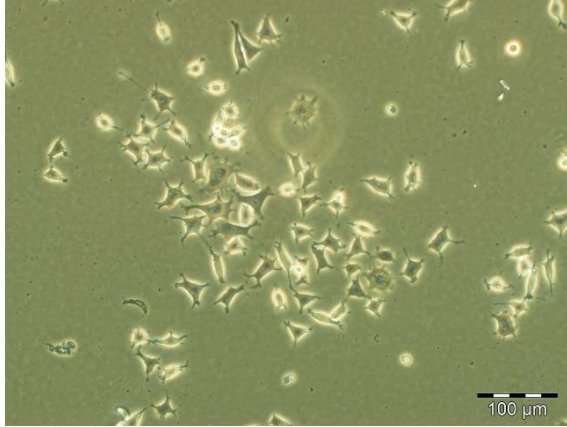
A. Non-coating



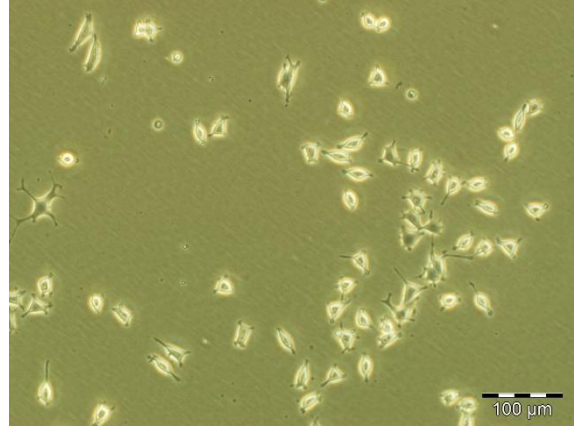
B. Collagen I



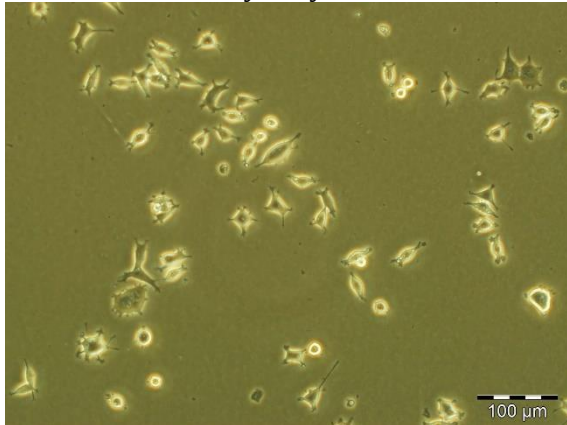
C. Collagen IV



D. Poly-D-lysine



E. Poly-L-lysine



F. Laminin

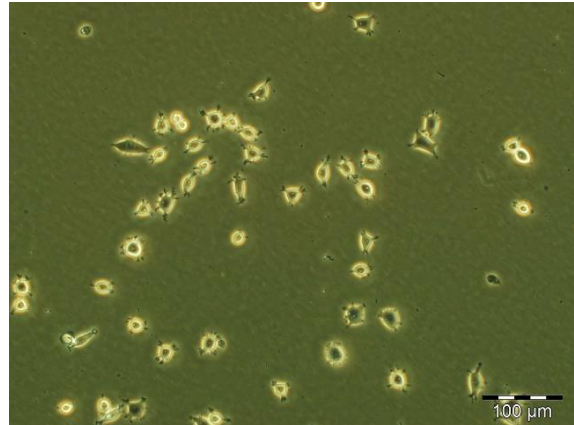
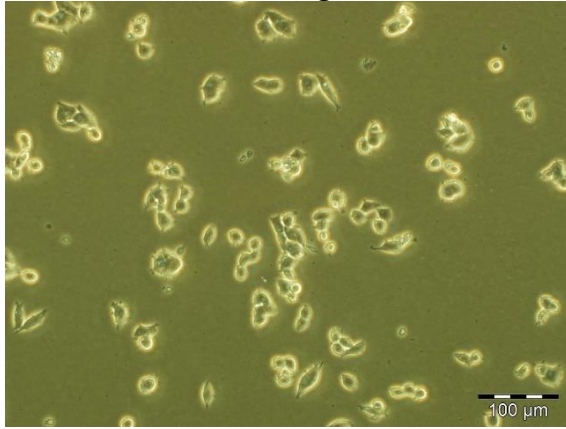


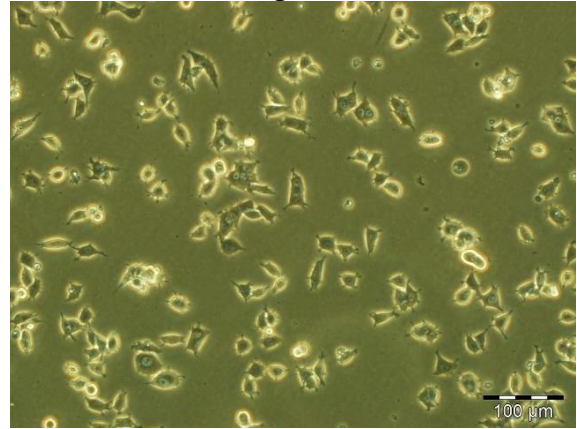
Figure 3.4: Microscopic images of NS-1 cells at 200x magnification in different adhesive substrates in DMEM high glucose 17.5% on day 1 after seeding. In comparison, collagen IV-coated flask (C) had better morphology than non-coated and other coated-flasks (A, B, D, E and F). Majority of the cells were spread out in the collagen IV-coated flask(C). Cells in non-coated flask (A), collagen I (B), poly-D-lysine (D), poly-L-lysine (E) and laminin (F) were smaller, brighter and less spread out.

Full serum

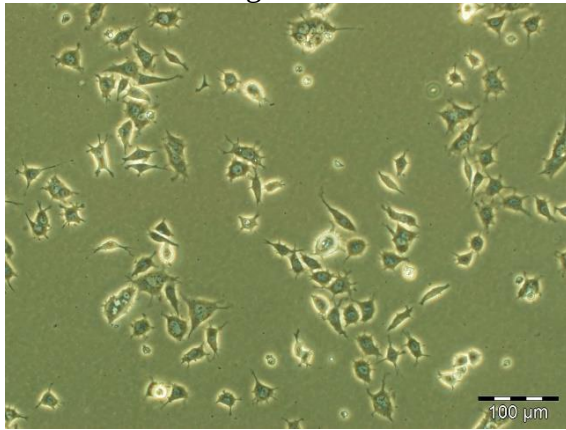
A. Non-coating



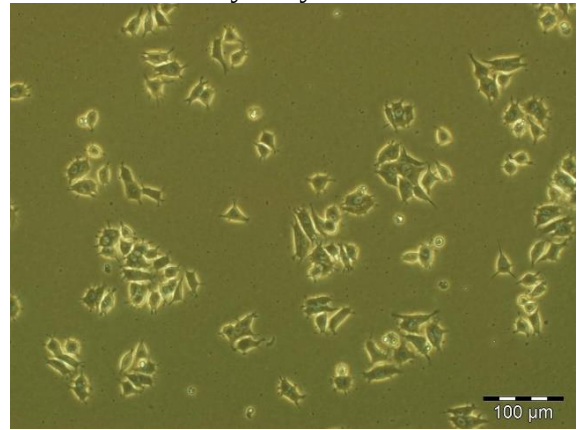
B. Collagen I



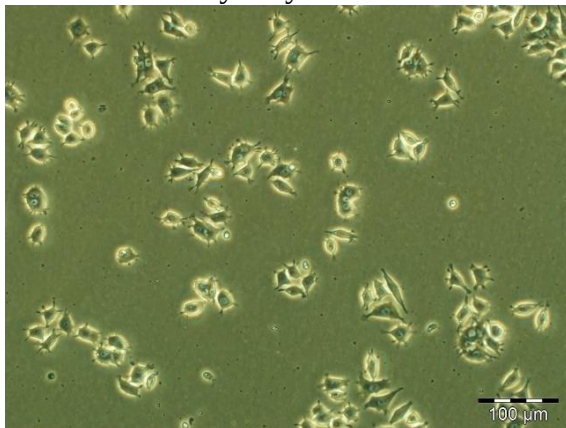
C. Collagen IV



D. Poly-D-lysine



E. Poly-L-lysine



F. Laminin

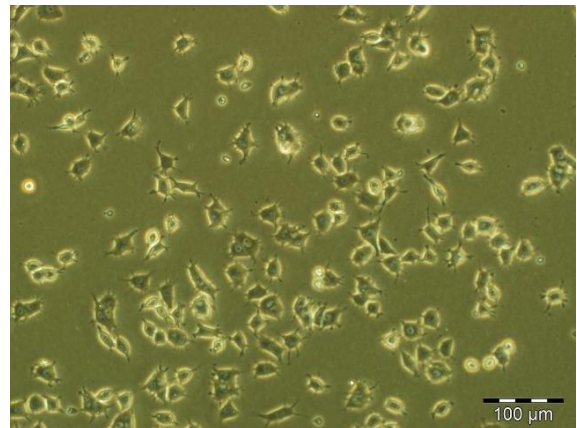


Figure 3.5: Microscopic images of PC12 cells (Riken) at 200x magnification in different adhesive substrates in DMEM high glucose 17.5% serum on day 1 after seeding. In comparison, coated-flask (B, C, D, E and F) had better morphology than non-coated flask (A). Cells were more spread out in coated-flask. Cell morphology was similar in all adhesive substrates.

Serum-free Supplemented DMEM High Glucose

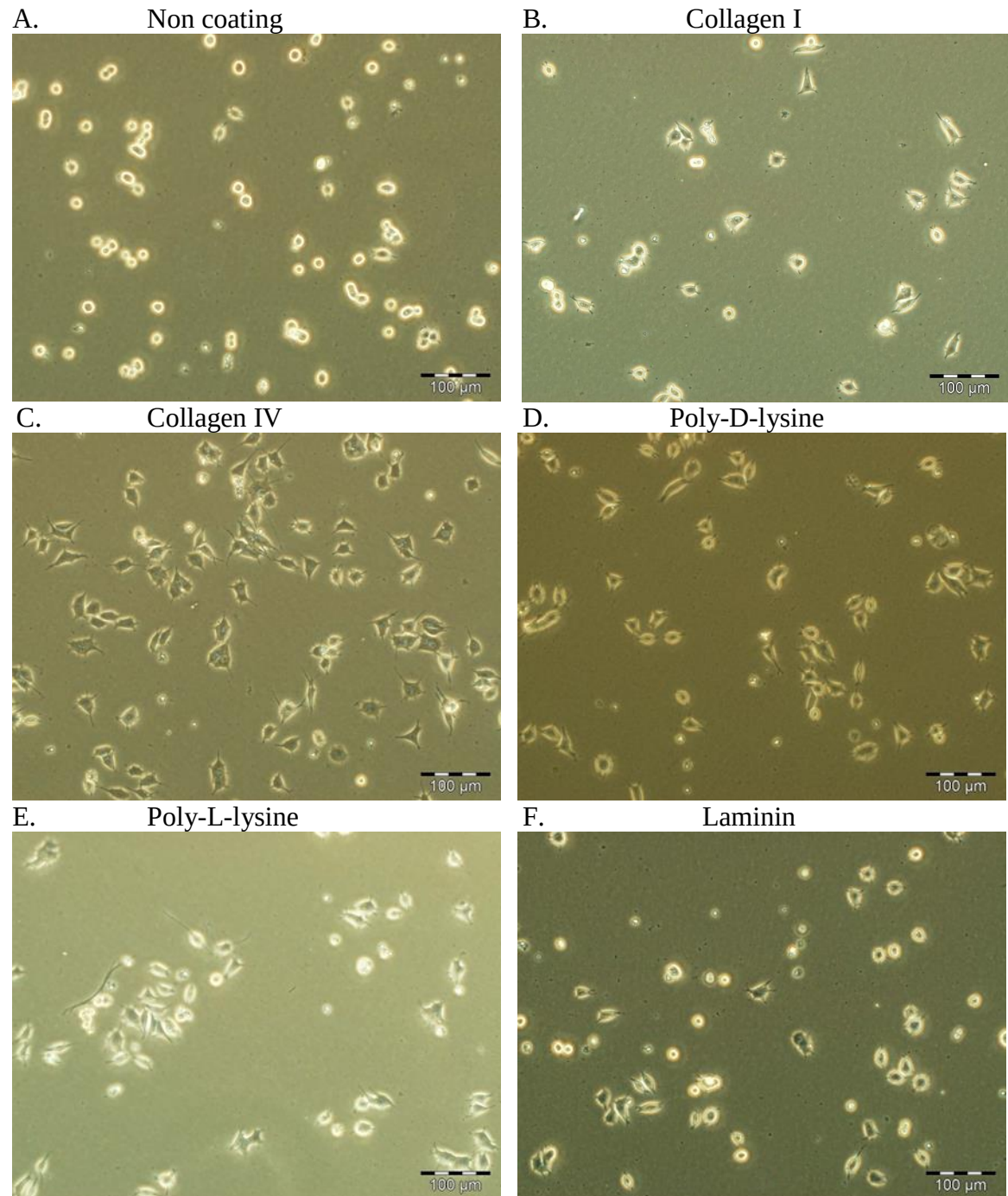


Figure 3.6: Microscopic images of PC12 cells (Riken) at 200x magnification in different adhesive substrates in serum-free supplemented DMEM high glucose on day 1 after seeding. All cells in non-coated flask showed rounded cell and not adhered to the flask (A). In comparison, collagen type-IV coated flask (C) had better morphology than other coated flasks (B, D, E, and F). Majority of the cells were spread out in the collagen IV-coated flask (C). Cells in collagen type I (B), poly-D-lysine (D), poly-L-lysine (E) and laminin (F) were smaller, brighter and less spread out.

Full Serum

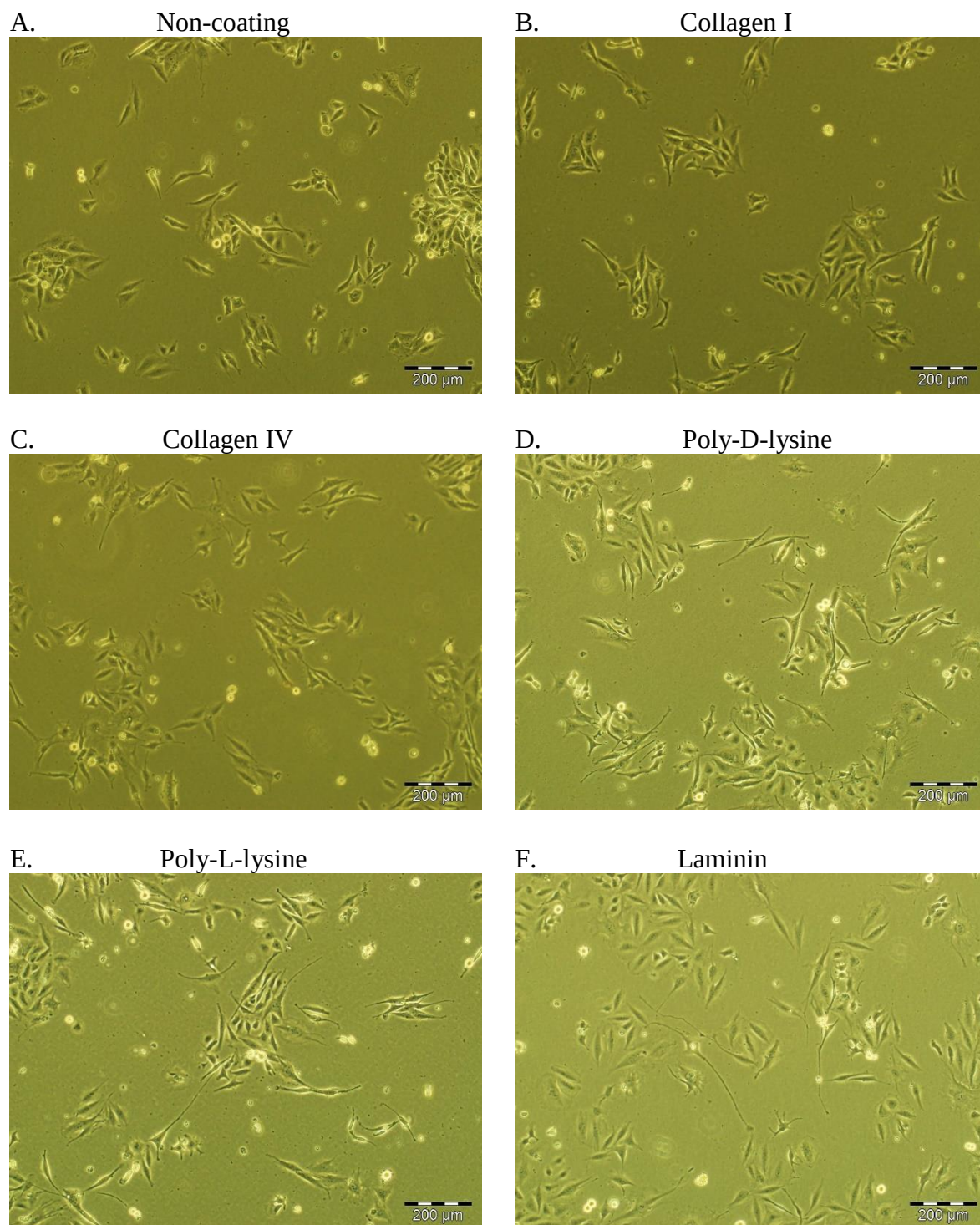
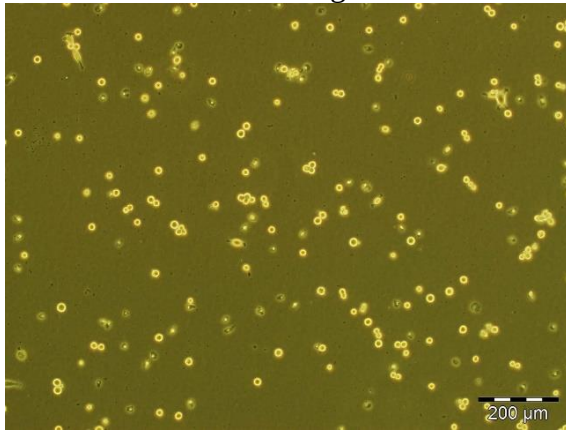


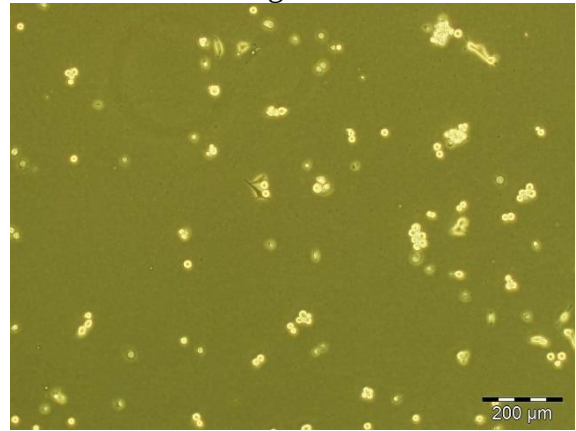
Figure 3.7: Microscopic images of PC12 Adh cells at 100x magnification in different adhesive substrates in F-12K 17.5% serum on day 1 after seeding. Cell morphology was similar in all adhesive substrates and non-coated flasks.

Serum-free Supplemented

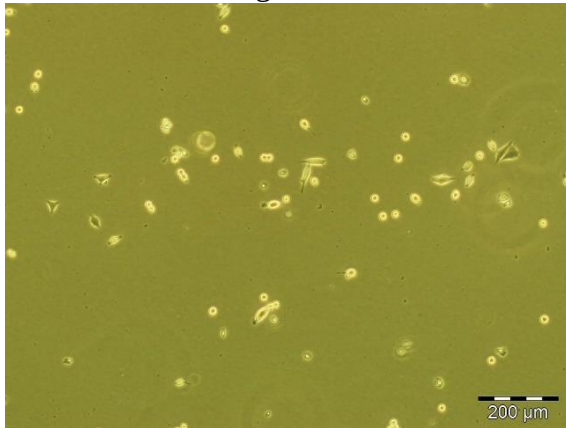
A. Non-coating



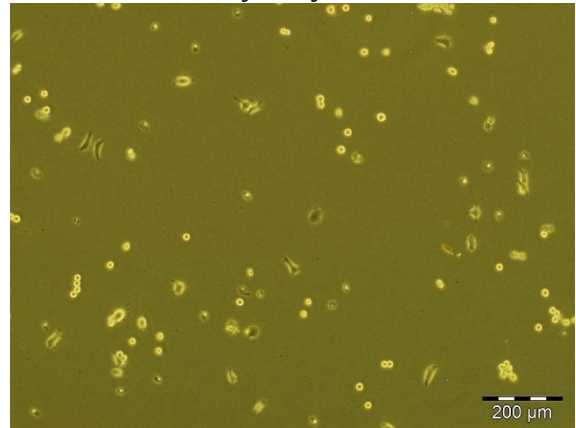
B. Collagen I



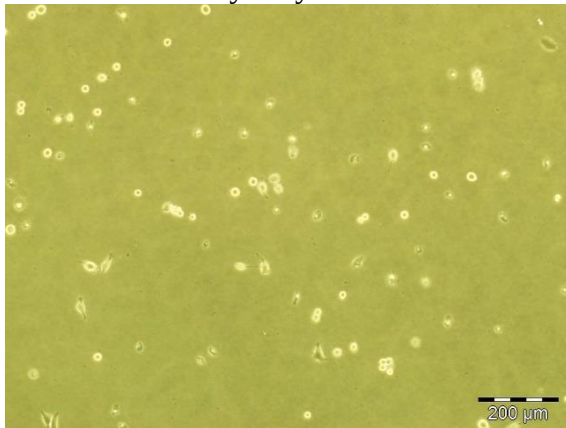
C. Collagen IV



D. Poly-D-lysine



E. Poly-L-lysine



F. Laminin

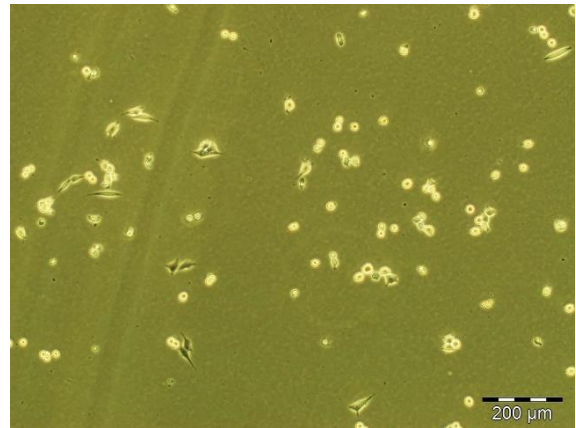


Figure 3.8: Microscopic images of PC12 Adh cells at 100x magnification in different adhesive substrates in serum-free supplemented F12-K on day 1 after seeding. Cells were unable to adhere to all the adhesive substrates and non-coated flasks.

3.3 Adaptation to Serum-free Supplemented Media for Three Cell Variants

3.3.1 PC12 Adh Cells

PC12 Adh cells failed to be adapted to F-12K serum-free media supplemented with six supplements (insulin, apo-transferrin, BSA, ethanolamine, β -mercaptoethanol, sodium selenite) as mentioned in Section 2.4.2. Figure 3.8 above showed that in serum-free supplemented F-12K media, PC12 Adh cells could not adhere and were rounded compared to cells in F-12K media with 17.5% serum as shown in Figure 3.1A. The experiment was repeated three times.

3.3.2 PC12 Cells (Riken)

PC12 cells (Riken) were successfully adapted to serum-free supplemented DMEM high glucose media on coated flask with the best morphology on collagen IV-coated flask as shown in Figure 3.6C. Cell morphology was the normal polygonal shape and similar to PC12 cells (Riken) in DMEM high glucose 17.5% on collagen IV-coated flask as shown in Figure 3.5C. The experiment was repeated three times.

3.3.3 Neuroscreen-1 (NS-1) Cells

NS-1 cells were successfully adapted to serum-free supplemented DMEM high glucose media on collagen IV-coated flask. Cell morphology in serum-free supplemented DMEM high glucose media was similar to cells growing in DMEM high glucose 17.5% serum (15% HS, 2.5% FBS) (Figure 3.9). The experiment was repeated three times.

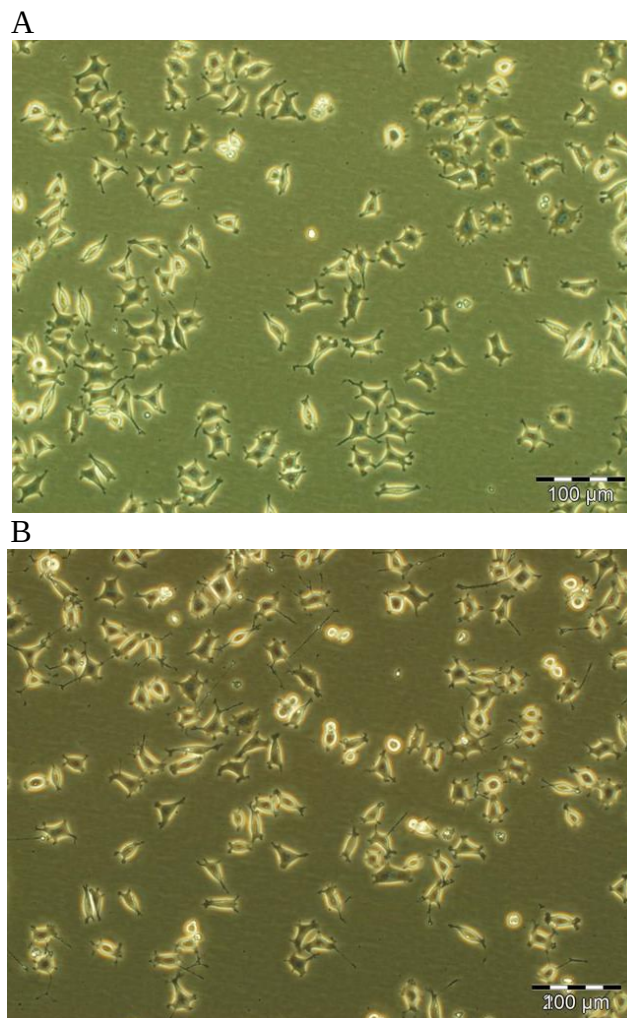


Figure 3.9: Microscopic images (200x) of NS-1 cells growing on collagen IV-coated flask in DMEM high glucose 17.5% serum (A) and DMEM high glucose serum-free supplemented media (B).

3.4 Summary of Optimisation of Cell Culture Conditions (Media and Adhesive Substrates) and Growth in Serum-free Supplemented Media

Table 3.1 summarized the findings of optimisation of cell culture media, adhesive substrates and the ability to grow in serum-free supplemented media for all 3 PC12 variants. PC12 Adh cells had similar cell morphology in all 3 media (F12K, DMEM and RPMI) and in all the substrates. PC12 cells (Riken) and NS-1 cells preferred DMEM high glucose and collagen IV as their media and substratum. PC12 Adh was not able to grow in serum-free supplemented media whereas PC12 cells (Riken) and NS-1 cells could grow well in serum-free supplemented media.

Table 3.1: Summary of optimisation of cell culture conditions (media and adhesive substrates) and growth in serum-free supplemented media.

	PC12 Adh	PC12 cells (Riken)	NS-1 cells
Culture media	Similar	DMEM high glucose	DMEM high glucose
Adhesive substrates	Similar	Collagen IV	Collagen IV
Serum-free supplemented media	Unable to grow	Able to grow	Able to grow

3.5 Comparison of the Dose Response to NGF in Three PC12 Variants

To determine the optimal concentration required to induce differentiation in PC12 Adh cells, PC12 cells (Riken) and NS-1 cells, the cells were treated with various concentrations of NGF (0 ng/mL, 50 ng/mL, 150 ng/mL and 300 ng/mL) for 4 days (Das, Freudenrich, & Mundy, 2004; Naranjo-Suárez et al., 2003; Yin et al., 2015). Cells were cultured in the optimised media and adhesive substrates (Table 3.1). The medium was changed and NGF was added every 2 days.

3.5.1 PC12 Adh Cells

Figure 3.10 shows representative images of PC12 Adh cells including untreated control (Figure 3.10A) and different dosages of NGF-treated cells (Figure 3.10B, 3.10C, 3.10D) after 4 days of differentiation. Figure 3.11 shows the percentage of the neurite-bearing cell on day 4 after differentiation with different dosages of NGF. The experiment was repeated 3 times. The highest percentage of differentiated cells was $6.9 \pm 0.8\%$ after 4 days of differentiation with 150 ng/mL NGF. Other micrographs of NGF-induced differentiation from Day 1 to Day 4 of PC12 Adh cells were shown in Appendix A1.

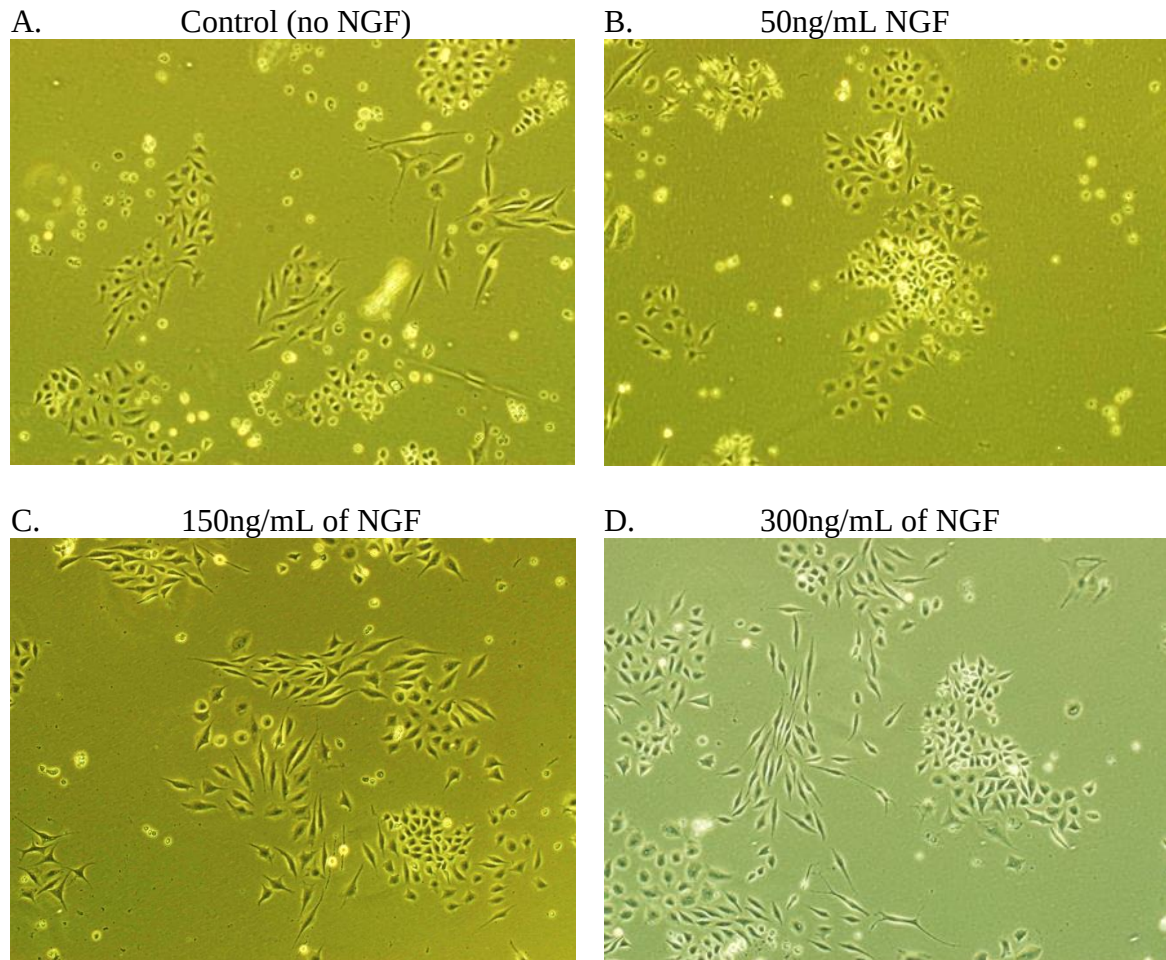


Figure 3.10: Microscopic images (100x) of neurite outgrowth of PC12 Adh cells treated with varying concentrations of NGF after 4 days of differentiation.

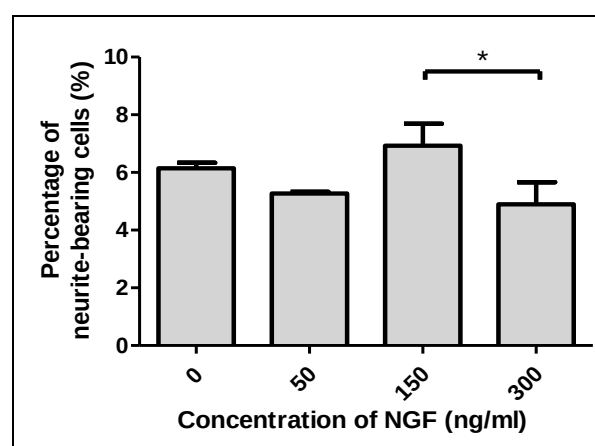


Figure 3.11: Percentage of neurite-bearing cells of PC12 Adh cells in different concentrations of NGF (ng/mL) after 4 days of differentiation. Each bar represents mean $\pm$ SD from 3 experiments. One-way ANOVA followed by post hoc Tukey's test was performed showing no significant differences between the concentrations of NGF except for 150 ng/mL vs 300 ng/mL, $*p < 0.05$).

3.5.2 PC12 Cells (Riken)

Figure 3.12 shows representative images of PC12 cells (Riken) with control (non-treated) (Figure 3.12A) and treatment with different dosages of NGF (Figure 3.12B, 3.12C, 3.12D) cells after 4 days differentiation. Cells extended neurites and the proportion of cells with extended neurites increased with the concentration of NGF. Figure 3.13 shows the percentage of the neurite-bearing cells on day 4 of differentiation with different dosages of NGF. The mean of the highest percentage of the differentiated cells was $36.0 \pm 5.6\%$ on day 4 using 300 ng/mL of NGF. Treatment with NGF produced a concentration-dependent increase in neurite outgrowth measured in terms of percentage of neurite-bearing cells. Other micrographs of NGF-induced differentiation from Day 1 to Day 4 of PC12 cells (Riken) were shown in Appendix A2.

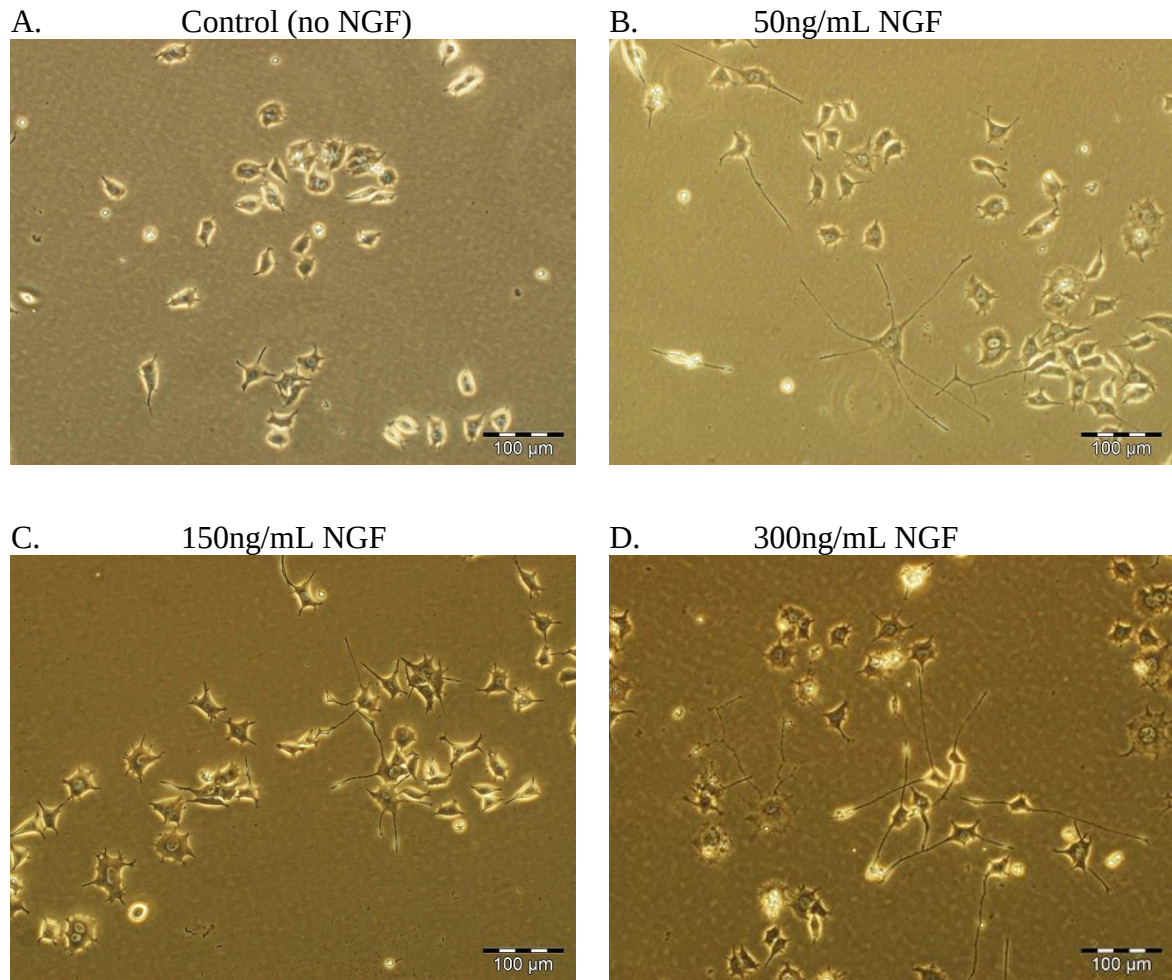


Figure 3.12: Microscopic images (200x) of neurite outgrowth of PC12 cells (Riken) treated with varying concentrations of NGF after 4 days of differentiation.

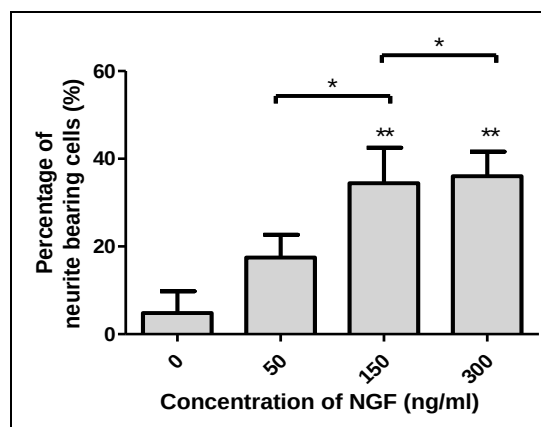


Figure 3.13: Percentage of neurite-bearing cells of PC12 (Riken) in different concentration of NGF (ng/mL) after 4 days of differentiation. Each bar represents mean $\pm$ SD from 3 experiments. One-way ANOVA followed by post hoc Tukey's test was performed showing

significant differences between 150 ng/mL vs 0 ng/mL (** $p < 0.01$), 300ng/mL vs 0ng/mL (** $p < 0.01$), 50 ng/mL vs 150 ng/mL (* $p < 0.05$), 150 ng/mL vs 300 ng/mL (* $p < 0.05$).

3.5.3 Neuroscreen-1 (NS-1) Cells

Figure 3.14 shows representative images of untreated NS-1 cells (Figure 3.14A) versus treatment with different dosages of NGF (Figure 3.14B, 3.14C, 3.14D) cells after 4 days of differentiation. Neurite-bearing cells were widespread after NGF. Extensive neurite networks were formed in both 150 ng/mL NGF and 300 ng/mL NGF, resembling typical neuronal morphology. Figure 3.15 shows the percentages of the neurite-bearing cells on day 4 after differentiation with different dosages of NGF. The mean of the highest percentage of the differentiated cell was $72.7 \pm 9.1\%$ on day 4 using 300 ng/mL of NGF. Treatment with NGF produced a concentration-dependent increase in neurite outgrowth measured in terms of percentage of neurite-bearing cells. Other micrographs of NGF-induced differentiation from Day 1 to Day 4 of NS-1 cell were shown in Appendix A3.

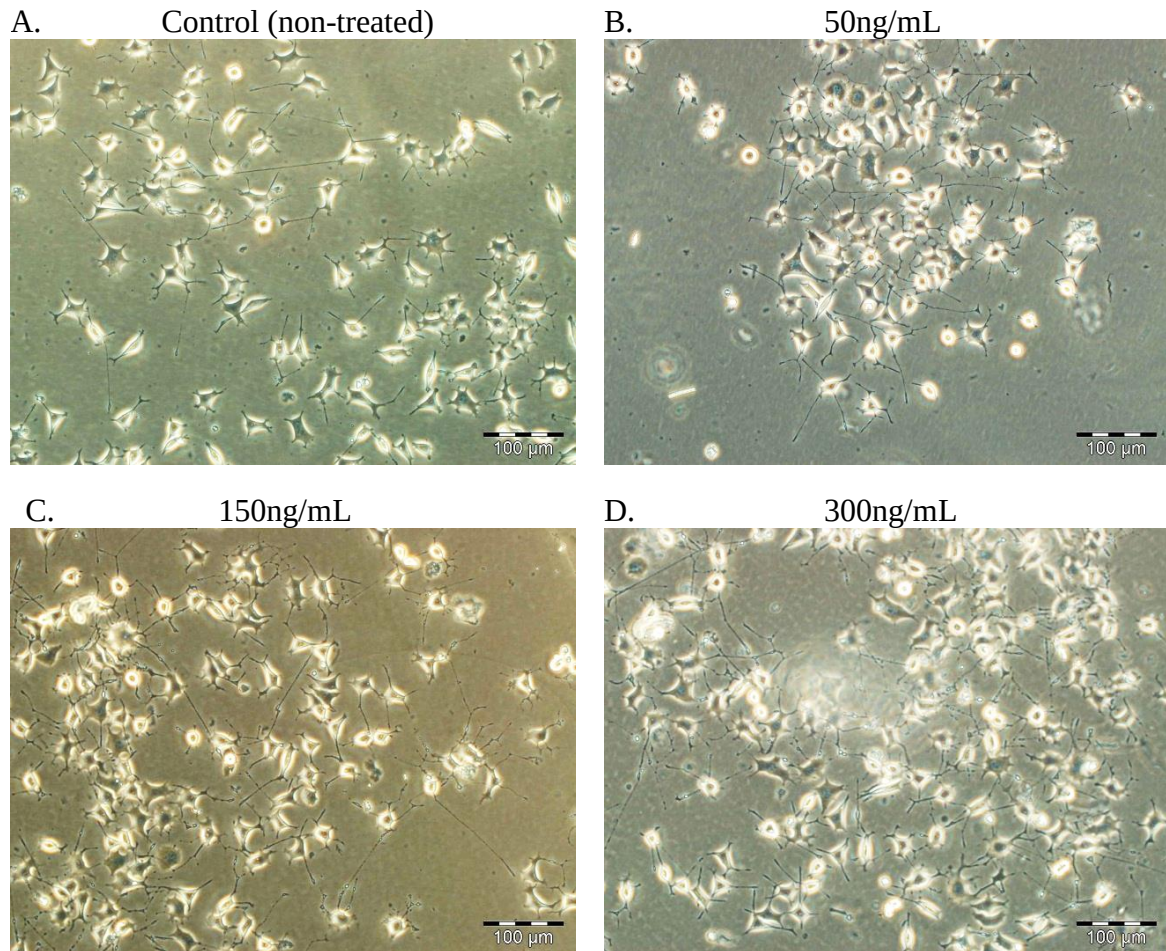


Figure 3.14: Microscopic images (200x) of neurite outgrowth of NS-1 cells treated with varying concentrations of NGF after 4 days of differentiation.

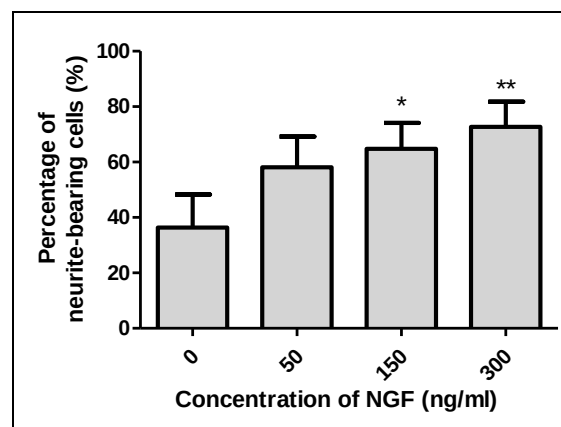


Figure 3.15: Percentage of neurite-bearing cells of NS-1 in different concentration of NGF (ng/mL) after 4 days of differentiation. Each bar represents mean values $\pm$ SD from 3 experiments. One-way ANOVA followed by post hoc Tukey's test was performed showing significant differences between 150 ng/mL vs 0 ng/mL ($*p < 0.05$) and 300 ng/mL vs 0 ng/mL ($**p < 0.01$).

3.6 Comparison of Three Cell Variants and Selection of the Most Suitable Cell Variant for *In Vitro* Model

Comparison of three cell variants by percentage of neurite-bearing cells showed that NS-1 cells had the highest response to NGF, followed by PC12 cells (Riken) and PC12 Adh cells. Figure 3.16 summarizes the comparison of percentage of neurite-bearing cells of three cell variants at different dosages of NGF after 4 days of differentiation. The results showed that the percentage of neurite-bearing cells for NS-1 cells and PC12 cells (Riken) were dose-dependent. Figure 3.17 shows the comparison of three cell variants highest percentage of neurite-bearing cells at day 4 of NGF (300 ng/mL)-induced differentiation. NS-1 cells had the highest response to NGF and were selected to use for the *in vitro* stroke model. Further optimisation of NS-1 cells was carried out to determine the optimal duration of NGF treatment and optimal concentration.

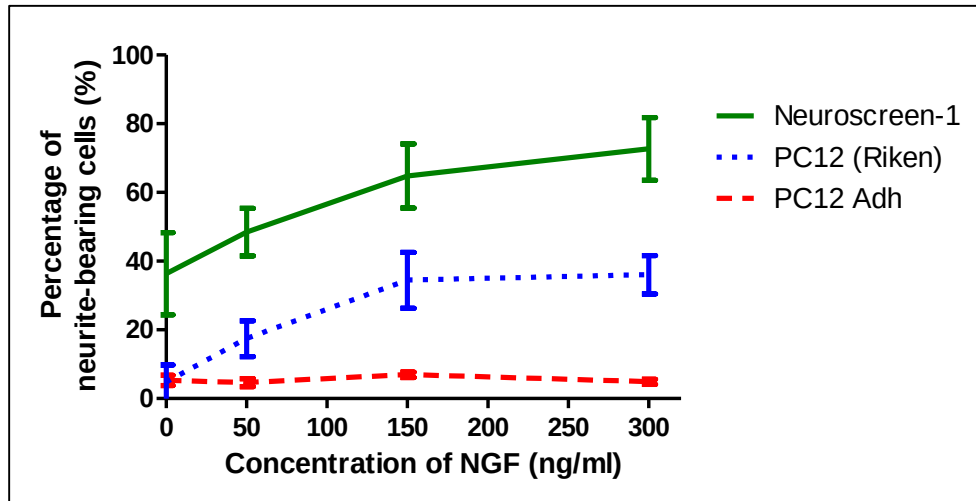


Figure 3.16: Percentage of neurite-bearing cells of 3 cell variants in increasing dosage of NGF after 4 days of differentiation. Data shown is the mean values $\pm$ SD of 3 independent experiments.

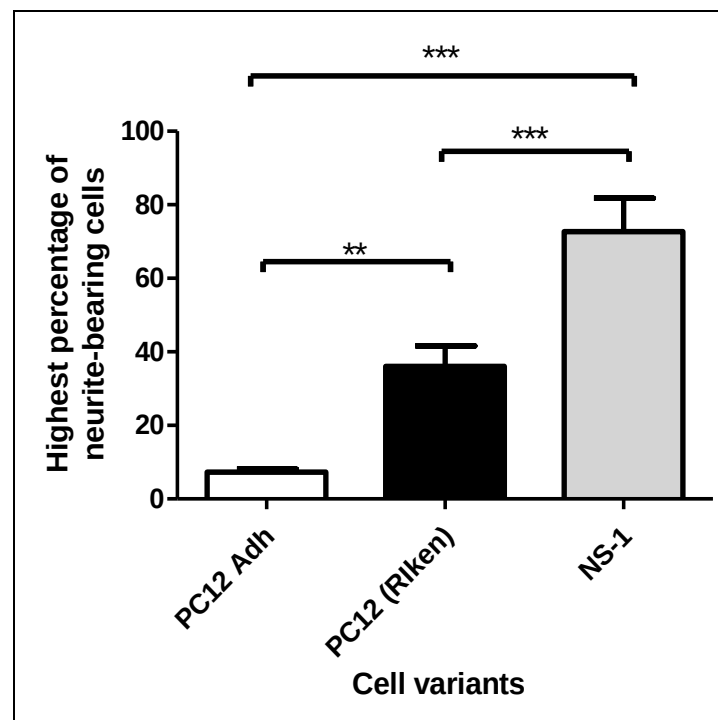


Figure 3.17: Highest percentage of neurite-bearing cells of 3 cell variants after 4 days of NGF-induced differentiation. Each bar represents the mean values $\pm$ SD of 3 independent experiments. One-way ANOVA followed by post hoc Tukey's test showed significant differences between all 3 cell variants: PC12 Adh cells vs PC12 cells (Riken) (** $p < 0.01$), PC12 cells (Riken) vs NS-1 cells (** $p < 0.001$), PC12 Adh cells vs NS-1 cells (** $p < 0.001$).

3.7 Optimisation of Nerve Growth Factor-induced Differentiation of Neuroscreen-1 (NS-1) Cells

NS-1 cells were optimised for use as an *in vitro* stroke model by determining the most suitable concentration of NGF and duration of differentiation. PC12 cells have been shown to reach a plateau in proportion of differentiated cells after 7 days of differentiation, with 75% differentiation by 4 days (Das, Freudenrich, & Mundy, 2004). Hence, we had differentiated PC12 cells for 4 days before assaying. However, NS-1 cells were reported to require a shorter time to reach maximal differentiation compared to another PC12 variant (Chaurasiya, Shukla, & Tekwani, 2017). As neurite-bearing cells was reported to need up to 3 days of differentiation to be noticeable (Teng et al., 2006), we compared the proportion of neurite-bearing cells in increasing doses of NGF after 3 and 4 days of differentiation. Figure 3.18 showed there was no significant differences between the proportions of neurite-bearing cells after 3 and 4 days of differentiation ($p > 0.05$, unpaired t-test). Hence 3 days was selected for the duration of differentiation in NS-1 cells. Table 3.2 showed the mean $\pm$ SD value (day 3 and day 4) and unpaired t-test p value for all concentration of NGF.

The NGF dose response data in Figure 3.19 was rearranged to exclude differentiation data after 4 days and added with 2 further doses of NGF for a fuller NGF dose range. Figure 3.19 shows both 150 ng/mL and 300 ng/mL of NGF gave the comparable maximal differentiation, 150 ng/mL of NGF was then used in subsequent experiments as it was more cost-effective.

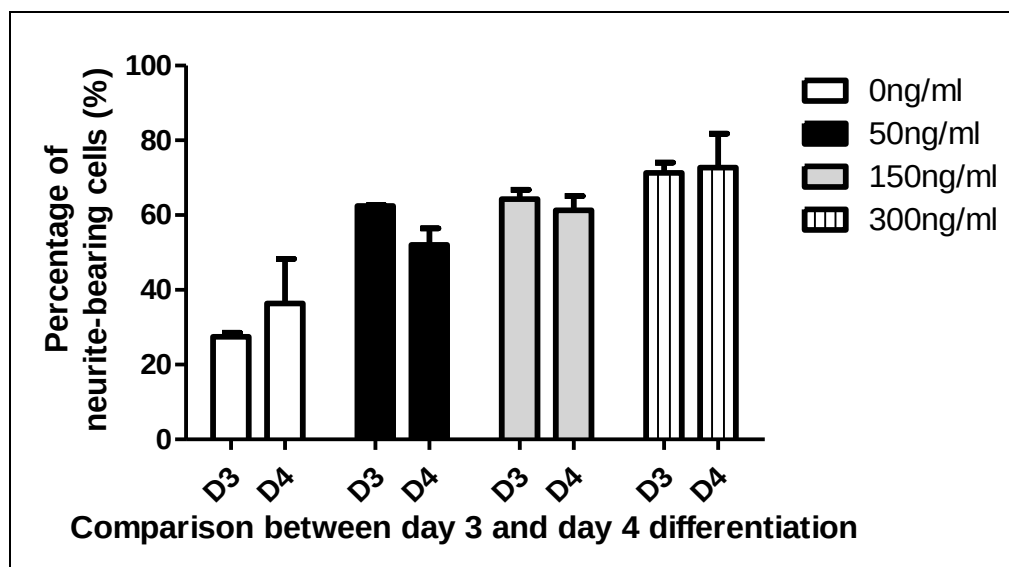


Figure 3.18: Percentage of neurite-bearing cells after 3 and 4 days of differentiation at increasing concentrations of NGF. There were no significant differences between day 3 and day 4 in terms of percentage of neurite-bearing cells for all concentration of NGF (0 ng/mL, 50 ng/mL, 150 ng/mL and 300 ng/mL) ($p > 0.05$, unpaired t-test). Each bar represents mean $\pm$ SD of 3 independent experiments.

Table 3.2: Mean $\pm$ SD value (day 3 and day 4) and unpaired t-test p value for all concentrations of NGF.

Concentration of NGF (ng/mL)	Mean $\pm$ SD (Day 3)	Mean $\pm$ SD (Day 4)	Unpaired t-test
0	27.43 $\pm$ 0.65	36.36 $\pm$ 5.97	$p = 0.2635$
50	62.48 $\pm$ 0.14	52.02 $\pm$ 3.13	$p = 0.0789$
150	68.87 $\pm$ 4.71	61.33 $\pm$ 2.21	$p = 0.2208$
300	71.29 $\pm$ 1.60	72.67 $\pm$ 5.25	$p = 0.8134$

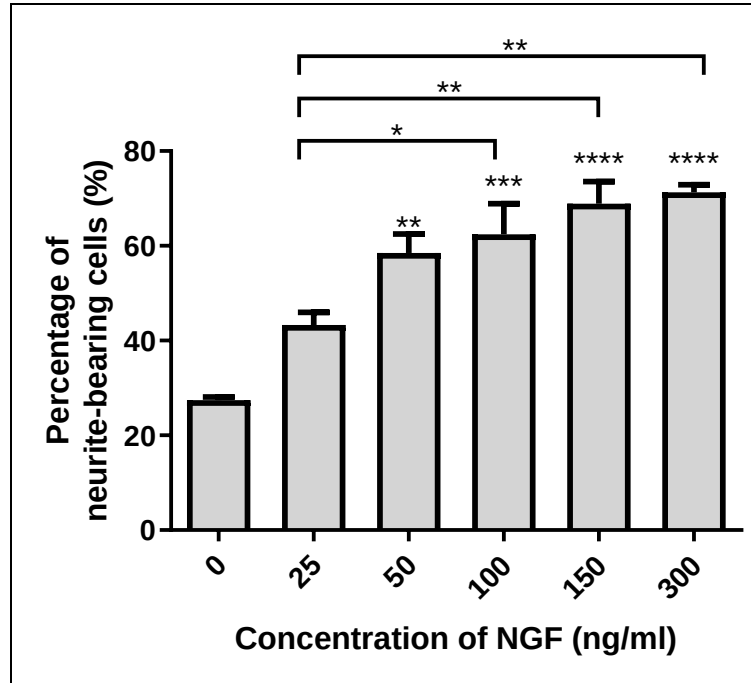


Figure 3.19: Percentage of neurite-bearing cells in range of NGF concentrations (ng/mL) after 3 days of differentiation. There were significant differences between 50 ng/mL vs. 0 ng/mL ($*p < 0.01$), 100 ng/mL vs. 0 ng/mL ($***p < 0.001$), 150 ng/mL vs. 0 ng/mL ($****p < 0.0001$) and 300 ng/mL vs. 0 ng/mL ($****p < 0.0001$). There were also significant differences between 25 ng/mL vs. 100 ng/mL ($*p < 0.05$), 25 ng/mL vs 150 ng/mL ($**p < 0.01$) and 25 ng/mL vs. 300 ng/mL ($**p < 0.01$). Each bar represents mean $\pm$ SD of 3 independent experiments.

3.8 Optimisation of Oxygen Glucose Deprivation (OGD) Protocol and Duration of OGD to Induce Cell Injury

3.8.1 Optimisation of Duration of Oxygen Glucose Deprivation (OGD) using Cell Viability Assay (MTT assay)

MTT assay was used to measure the cell viability after OGD. The formula of the percentage of viable cells is as below.

$$\text{Percentage of viable cells} = \frac{\text{Absorbance at 570 nm of experimental groups}}{\text{Absorbance at 570 nm of control groups}} \times 100\%$$

Figure 3.20 shows the percentage of viable differentiated NS-1 cells after different durations of OGD. The results showed that the percentage of viable cells significantly decreased ($***p < 0.001$) after 1-4.5 hours of OGD and further decreased after 6 hours of OGD. After 1, 2, 3, 4.5 and 6 hours of OGD, the percentage of viable cells were $72.9 \pm 1\%$, $74.0 \pm 7.6\%$, $66.6 \pm 3.6\%$, $72.9 \pm 7.4\%$ and $50.7 \pm 2.0\%$ respectively (mean $\pm$ SD of 3 experiments).

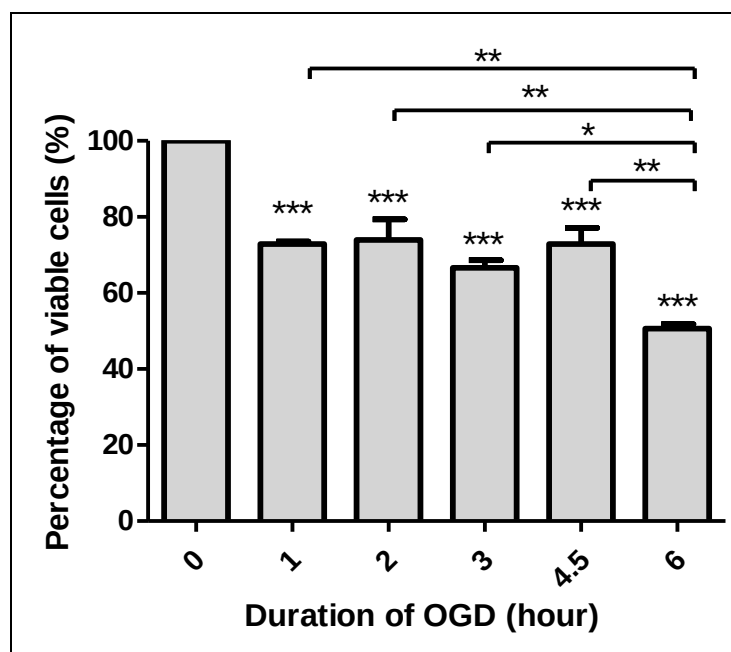


Figure 3.20: Percentage of viable cells determined by MTT assay after different durations of OGD. Data is mean $\pm$ SD of 3 independent experiments carried out in triplicate. One-way ANOVA followed by post hoc Tukey's test was performed showing significant differences between the OGD-treated groups and control (0-hour OGD, *** $p < 0.001$). There were significant differences between 1-hour vs. 6-hour (** $p < 0.01$), 2-hours vs. 6-hour (** $p < 0.01$), 3-hour vs. 6-hour (* $p < 0.05$) and 4.5-hour vs. 6-hour (** $p < 0.01$).

3.8.2 Optimisation of Duration of Oxygen Glucose Deprivation (OGD) using Apoptosis Assay (Caspase 3/7 Assay)

Figure 3.21 shows caspase 3/7 activity in differentiated NS-1 cells after different durations of OGD. Data shows fold-increase in luminescence signal over control (0-hour OGD). The increase in cell apoptosis occurred significantly after 2 hours and continue to increase after 4.5 hours of OGD. The fold increase of caspase 3/7 activity for 1, 2, 3, 4.5 and 6 hours of OGD were 1.5 ± 0.1 , 3.3 ± 0.7 , 4.1 ± 0.4 , 4.3 ± 0.4 and 4.1 ± 0.7 respectively.

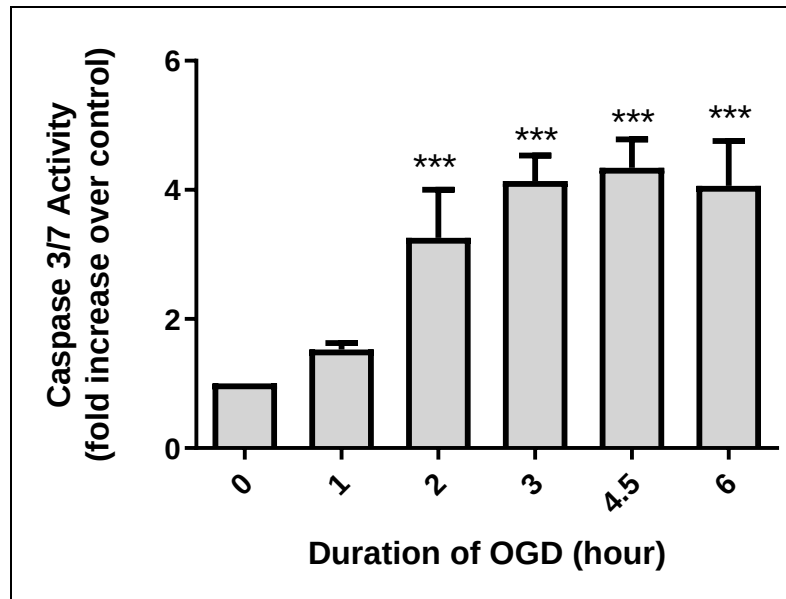


Figure 3.21: Caspase 3/7 activity after different durations of OGD. Data are mean $\pm$ SD of 3 independent experiments carried out in triplicate. One-way ANOVA followed by post hoc Tukey's test was performed showing significant differences between the 2, 3, 4.5 and 6-hour OGD-treated group over control (0-hour OGD, *** $p < 0.001$). There was no significant difference between 2-hour and 3-hour OGD ($p > 0.05$).

3.9 Optimisation of Duration of Oxygen Glucose Deprivation (OGD) for *In Vitro* Stroke Model

Comparing the optimisation of duration of OGD by using both cell viability and caspase 3/7 assays suggested that 3 hours as the optimal duration of OGD. Three hours was the shortest duration to reach the maximal caspase 3/7 activity while cell viability assay showed that there were sufficient viable cells to support recovery. Therefore, 3 hours of OGD was selected for *in vitro* stroke model.

3.10 Validation of the *In Vitro* Stroke Model using the Neuroprotectant 8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT)

Differentiated NS-1 cells undergoing OGD were subsequently treated with either 8-OH-DPAT (5HT_{1A} receptor agonist) or WAY 100635 (5HT_{1A} receptor antagonist). Figure 3.22 shows that caspase 3/7 activity of 8-OH-DPAT-treated NS-1 cells was significantly reduced compared to the untreated cells ($p < 0.01$). When cells were treated with WAY 100635, caspase 3/7 activity did not reduce significantly compared to untreated cells ($p > 0.05$). Hence, 8-OH-DPAT was also neuroprotective in NS-1 cells. This effect was not seen with WAY 100635 which binds to the same receptors without activating them.

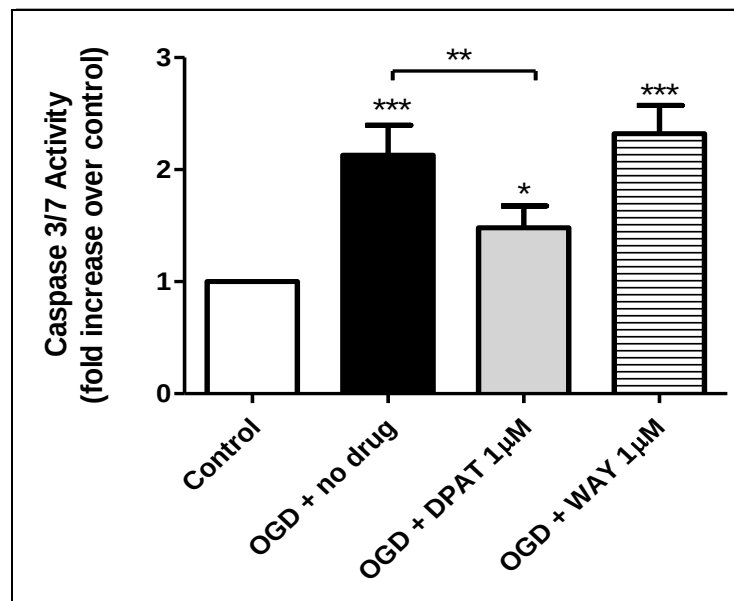


Figure 3.22: Effect of 8-OH-DPAT and WAY 100635 on apoptotic NS-1 cells following OGD. Data is expressed as mean and $\pm$ SD. Experiment was repeated for 8-OH-DPAT (n=3) and WAY (n=2) in triplicate. One-way ANOVA followed by post hoc Tukey's test was performed showing significant differences between OGD + DPAT 1 μ M vs. OGD + no drug (** $p < 0.01$) and no significant differences between OGD + WAY 1 μ M vs. OGD + no drug ($p > 0.05$). There were significant differences between OGD + no drug vs. control (*** $p < 0.001$), OGD + DPAT 1 μ M vs. control (* $p < 0.05$) and OGD + WAY 1 μ M vs. control (*** $p < 0.001$).

CHAPTER 4

DISCUSSION

4.1 Comparison and Selection of Suitable Culture Media of Three Cell Variants

Cell culture media was supplemented with 17.5% serum (15% horse serum and 2.5% fetal bovine serum) as recommended by supplier ATCC for PC12 Adh cells. PC12 Adh cells were able to grow in all three culture media (DMEM high glucose, F-12K and RPMI) in standard plastic cell culture flasks. F-12K medium was selected as it was the recommended medium used by the supplier (ATCC) and it has been used by researchers in cell differentiation (Eik et al., 2012; Yin et al., 2015). PC12 Adh cells could grow well in all the media tested, likely due to its ability to adhere to the flask well without any added coating. Good cell adherence is a characteristic of this cell line described by its supplier ATCC.

PC12 cells (Riken) was not able to adhere and grow in RPMI medium on a standard normal plastic flask. Cells were clumped up and floating. A similar result was reported in another comparative study of PC12 cells where two PC12 cell lines from different laboratories (gifts from other laboratories) lost their ability to adhere to flasks when cultured in RPMI medium (Dixon et al., 2005). Our finding suggests that RPMI is not a suitable medium for PC12 cells (Riken). In comparison, PC12 cells (Riken) could grow well in DMEM high glucose, with normal cell morphology. In comparison, PC12 Adh cells had better cell adherence compared to PC12 cells (Riken). In the normal plastic flask, PC12 cells (Riken) could not adhere and grow in RPMI but PC12 Adh cells could adhere and grow in all culture media (DMEM high glucose, RPMI and F-12K) in normal plastic flask.

NS-1 cells, a variant of PC12 cell line was originally cultured in RPMI 17.5% serum and successfully adapted to DMEM high glucose 17.5% serum. Comparison of the morphology of the cells in both media showed that DMEM high glucose was superior to RPMI. In RPMI medium, significant visible cell debris and floaters were observed. Also, most of the cells in RPMI were brighter compared to cells in DMEM high glucose where cells looked darker (phase-dark cells indicate firm adherence) (Milner & Campbell, 2002) and well spread out. This suggested that the cells in RPMI did not adhere to the flask as well as in DMEM high glucose. In DMEM high glucose, cells were well spread out and some were starting to extend short neurites. This showed that the cells adhered firmly to the flask.

DMEM high glucose is a preferred medium for both PC12 cells (Riken) and NS-1 cells. One possible reason is DMEM high glucose medium can increase cell adhesion because of its higher Ca^{2+} concentration compared to RPMI (Kemler, Ozawa, & Ringwald, 1989). DMEM high glucose medium contained 1.8 mM calcium but RPMI medium only contains of 0.4 mM calcium. Besides, higher magnesium content in DMEM high glucose (0.8 mM) than RPMI (0.4 mM) may also contribute to the better cell morphology as magnesium is required for attachment of PC12 cells (Turner, Flier, & Carbonetto, 1987).

In our study, DMEM high glucose was found to be a better medium in both PC12 cells (Riken) and NS-1 cells. DMEM high glucose is the medium for PC12 cell-based *in vitro* stroke model in several studies of neuroprotective agents (Guo et al., 2013; Mohammad Alizadeh et al., 2018; Tabakman, Jiang, Schaefer, Levine, & Lazarovici, 2004). One previous study also showed DMEM high glucose is a better culture medium for PC12 cells

in their comparison with mixture of Ham's F-12 and DMEM high glucose (1:1) (Sakagami et al., 2017). This finding suggests that the RPMI which was used by Greene and Tischler (Greene & Tischler, 1976) in the establishment of this cell line is not necessary the best medium for PC12 cells. Our finding of changes in cell morphology when PC12 cell variants are cultured in different media suggests that optimising cell culture medium for PC12 cell-based *in vitro* stroke model is necessary and important.

4.2 Serum-free Supplemented Media

In this study, we have successfully adapted both PC12 cells (Riken) and NS-1 cells to serum-free supplemented medium. PC12 cells (Riken) has been shown to be able to grow in hormonally defined serum-free media (Ohnuma et al., 2006). This study is the first report showing NS-1 cells to be able to grow in serum-free supplemented media. However, we failed to adapt PC12 Adh cells to serum-free supplemented media. Further investigations are needed to know the reasons for this.

The ability of PC12 cells (Riken) and NS-1 cells to grow in serum-free supplemented media shows that these two cell variants are suitable to be used for *in vitro* stroke model to test drug candidates which is advantageous to be carried out in serum-free media. Serum contains numerous unknown factors which varies from batch to batch (Cartwright & Shah, 2002). The usage of serum-free supplemented media during the screening of neuroprotective agents can avoid the effect of similar compounds present in the serum. Hence, this produces more reliable and consistent result. Many *in vitro* stroke models used serum contained media

for screening new neuroprotective agents. This can result in confounding effect on drug screening processes. Hence, the current *in vitro* stroke model which uses serum-free supplemented media is a better approach in drug screening process.

The PC12 cell line is increasingly being used in many fields besides screening for neuroprotective agents, including testing new materials (Okuyama et al., 2003) and brain implants in Parkinson's disease (Yoshida et al., 2003). To facilitate more precise studies, confounding unknown factors that may be present in the serum should be avoided (Ohnuma et al., 2006). Our finding will be beneficial for researchers who are interested to use the PC12 cell line and culture it in a serum-free media.

In addition, serum-free supplemented media was used to find out the best adhesive substrates for the PC12 cells. In this study, we removed the serum in testing the best adhesive substrates for PC12 cells (Riken) and PC12 Adh cells. As serum contains conditioning factors that aid in cell-substratum attachment (Brunner et al., 2010), the removal of serum causes cell attachment to be solely dependent on the substratum. Hence, the removal of the serum may reveal the preferential substratum of the cell variant. In this study, we have shown the differences of morphology of PC12 cells (Riken) in the different substrates in a serum-free supplemented media. This finding suggests that removal of serum in testing substrate can be a novel approach for testing substratum.

4.3 Comparison and Selection of Most Suitable Adhesive Substrates of 3 Cell Variants

PC12 Adh cells showed similar morphology in all the adhesive substrates in full serum F-12K after overnight incubation. There was no difference between the coated flask and non-coated flask in terms of morphology in full serum. According to ATCC, PC12 Adh cell variant is an adherent variant of PC12 cells which have been adapted to Corning CellBIND® flasks to improve cell attachment. This adherent variant has good cell attachment without adhesive substrates. In the serum-free supplemented medium, the cells were not able to adhere to the flask in all the adhesive substrates tested. The Adh cells were floating, with only a few adhered cells. This raises the questions of what are the factors needed for PC12 Adh cells to grow in the serum-free supplemented medium.

PC12 cells (Riken) had a similar morphology when they were cultured in different types of adhesive substrates in DMEM high glucose 17.5% serum after overnight incubation. Removal of serum was done to see whether there are any differences between the various adhesive substrates to support cell growth. Cell morphology in collagen IV-coated flask was better compared to the other adhesive substrates when they were cultured in serum-free supplemented DMEM high glucose medium. As a result, collagen IV was selected as the adhesive substrate for subsequent culture and experiments. Collagen IV was also reported to be superior to collagen I and poly-L-lysine for PC12 cells (Riken), supporting better PC12 cell morphology which were more spread out and firmly attached (Ohnuma et al., 2006). Greater cell attachment of PC12 cells (variants not mentioned) to the flask has also been shown in collagen IV-coated flask compared to collagen I-coated flask (Turner et al., 1987;

Vlodavsky, Levi, Lax, Fuks, & Schlessinger, 1982). Our finding suggests that Collagen IV is the most suitable coating for PC12 Riken cells to support cell adherence.

NS-1 cells had the best cell morphology when they were cultured in collagen IV-coated flask in full serum DMEM high glucose 17.5%. Cells looked more spread out compared to other adhesive substrates and non-coated flask. In other substrates majority of the cells were smaller and brighter by phase contrast microscopy. This morphological difference showed that the cells adhered better in collagen IV-coated flasks. To date, there is no comparison of adhesive substrates for NS-1 cells except for a preliminary data showing that the type of collagen did not affect the cell viability and neurite outgrowth (Radio, Breier, Shafer, & Mundy, 2008). However, collagen IV has been used in a recent study showing a good response to NGF differentiation (Chaurasiya et al., 2017) suggesting that collagen IV is suitable for supporting study of neurite outgrowth .

From this comparison of adhesive substrates, collagen IV is a suitable cell adhesive substrate for both PC12 cells (Riken) and NS-1 cells. NGF differentiation requires cells to be grown in an adhesive substrate. In one report, NGF differentiation required PC12 cells to be plated on a collagen IV-coated flask (Gordon et al., 2013). One of the possible reason that collagen IV is better than Collage I is that collagen IV is an exclusive member of the basement membranes compared to other types of collagen. Through a complex inter- and intramolecular interactions, collagen IV can form supramolecular networks that influence cell adhesion, migration, and differentiation (Khoshnoodi, Pedchenko & Hudson, 2008).

4.4 Comparison of Nerve Growth Factor (NGF)-induced Differentiation Response of Three PC12 Cell Variants

PC12 cells are able to differentiate into neuronal-like cells. The comparison of the 3 cell variants on the 4th day of differentiation showed that NS-1 cells had the highest percentage of neurite-bearing cells compared to another 2 PC12 variants. The percentage of neurite-bearing NS-1 cells with 300ng/mL of NGF was $72.7 \pm 9.1\%$ on day 4 of differentiation compared to $36.0 \pm 5.6\%$ for PC12 cells (Riken) (300ng/mL) and $6.9 \pm 0.8\%$ for PC12 Adh cells (150ng/mL). Hence, NS-1 cells is the most suitable PC12 cell variant for the establishment of *in vitro* stroke model which requires differentiation into neuronal cells.

In a study which compared PC12 cells from ATCC and NS-1 cells, NS-1 was reported to differentiate better with a lower dosage of NGF and shorter duration of differentiation (Chaurasiya et al., 2017). In our study, comparing NS-1 cells with PC12 cells (Riken), NS-1 cells also showed higher percentage of neurite-bearing cells with a lower NGF dosage and shorter duration of differentiation. On the 4th day of differentiation PC12 cells (Riken) had $36 \pm 5.6\%$ neurite-bearing cells with 300ng/mL NGF but for NS-1 cells, on day 3rd of differentiation, the percentage of neurite-bearing cell was already $68.9 \pm 8.2\%$ with 150ng/mL NGF.

PC12 Adh cells had the lowest response to NGF after 4 days of differentiation. Although few reports showed the ability of PC12 Adh cells to respond to NGF (Dikmen, 2017; Duan et al., 2012), studies on differentiation potential of PC12 Adh cells in response

to NGF are still limited. Discussion in online fora (Alexis, 2017, July 23) revealed that PC12 Adh cells has been found to be unable to differentiate into neuronal-like cells. Further investigations are needed on this PC12 variant to understand why it differentiates poorly. Different clones of PC12 cells have been reported to have differences in their ability to respond to NGF (Guroff, 1985). Greene and Tischler reported that the difficulty of PC12 cells to adhere to tissue culture flask (Greene & Tischler, 1976) can favour the continuous selection of a subclone that can adhere to the flask.

When changes in the characteristics of PC12 cells were first reported, the remedy proposed was to culture with the original media, sera and substrate in order to avoid selecting for clonal variants, especially those non-responsive to NGF (Teng et al, 2006). However, the high number of clonal variants now means there is no one standard culture condition. Instead, PC12 cells that are newly obtained or purchased need to be examined to ensure the phenotype is appropriate for the work it is intended for. It is important to know the maximal extent of differentiation possible for one's PC12 variant, especially if it is for an *in vitro* stroke model where the ability to differentiate is a key requirement. Although commercially supplied cells come with recommended culture conditions, in this study we show the need to optimise media and substrate. We optimised the culture conditions of their frequently used commercially available PC12 variants and demonstrate their variation in capacity for NGF-induced differentiation.

4.5 Optimisation of Oxygen Glucose Deprivation (OGD) Duration to Induce Cell Injury

Oxygen and glucose deprivation on NGF-differentiated PC12 cells simulates the condition of stroke. Ischaemic insults can cause both necrosis and apoptosis of brain cells (Martin et al., 1998). We optimised the duration of OGD by measuring cell viability and activity of apoptosis effector enzymes (caspase 3 and 7). We have found out that 3 hours of OGD was the optimum duration to simulate stroke in differentiated NS-1 cells as it is the shortest duration to reach the maximal caspase 3 and 7 activity. At 3 hours of OGD there were sufficient viable cells remaining to screen for neuroprotectants. This is in agreement with a published protocol which reported that the study the protective effect of an agent required an OGD duration which causes 40-50% cell death. This is because an extreme cell death (e.g. $\geq 80\%$) would cause difficulty in detecting the effect of protective agents (Ryou & Mallet, 2018). 3 hours of OGD significantly increased caspase 3 and 7 activity and decreased cell viability.

In this study, after 3 hours of OGD insult, the percentage of cell viability was 66.6% measured using MTT assay. This finding is similar to a report which showed that OGD duration of 3 hours resulted in around 65.7% of cell viability (Singh et al., 2009). Three hours of OGD resulted in an increase of around 4-fold in caspase 3 and 7 activity. Similar duration of 3 hours OGD to induce apoptosis was reported by other group testing potential neuroprotectants (Liang et al., 2013; Liu, Wang, Liu, Zhang, & Lou, 2013).

A typical duration of OGD that can cause adequate widespread cell death by apoptosis and necrosis for PC12 cell line ranges from 1 to 4 hours (Lahiani et al., 2018). In this study, we have shown that 3-hour OGD was sufficient to cause substantial apoptosis. This finding is useful for researchers who wish to use NS-1 cell line for studying neuroprotective agents. Different cell lines can have different responsiveness to OGD insult (Singh et al., 2009). To date, NS-1 cells are mostly used for neurite outgrowth assay but uncommon for *in vitro* stroke models.

4.6 Validation of the *In Vitro* Ischaemic Stroke Model by Testing the Neuroprotective Effect of 8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT)

In the validation of this *in vitro* model, 8-OH-DPAT, a known neuroprotectant which binds and activates 5HT_{1A} receptors significantly reduced caspase 3 and 7 activity following OGD. 5HT_{1A} receptors has been reported to promote survival in stressed cells (Singh, Chromy, Boyers, Dawson, & Banerjee, 1996). As a control to 1 μ M 8-OH-DPAT, 1 μ M WAY 100635 which binds to the same receptors (but does not activate the receptors) did not reduce caspase 3 and 7 activity. Hence this system was validated for its ability to screen potential neuroprotective agents.

8-OH-DPAT has shown neuroprotective properties in many animal models of central nervous system injury (Oosterink, Korte, Nyakas, Korf, & Luiten, 1998; Torup, Møller, Sager, & Diemer, 2000) and in other injury models (Ahlemeyer & Kriegelstein, 1997; Druse, Tajuddin, Gillespie, & Le, 2005). We found 8-OH-DPAT able to reduce caspase 3 and 7

activity in an *in vitro* stroke model at a concentration reported for inhibition of apoptosis in cultured neurons (Ahlemeyer & Krieglstein, 1997).

Although several studies have reported the neuroprotective properties of 8-OH-DPAT in *varies vivo* and *in vitro* models, this is the first report of its neuroprotective effect in PC12 cells. A possible mechanism for this neuroprotection effect could be the 8-OH-DPAT potentiating NGF-induced neurite outgrowth in PC12 cells, as previously reported (Ishima, Iyo, & Hashimoto, 2012). In a similar report, brexpiprazole, a partial agonist of 5-HT_{1A} receptors significantly potentiate NGF-induced neurite outgrowth in PC12 cells and the effect was blocked by WAY 100635 showing the effect was mediated by activation of 5-HT_{1A} receptor (Ishima et al., 2015).

Our finding of the neuroprotection effect of 8-OH-DPAT in PC12 cells provides the proof the concept that this *in vitro* stroke model is ready to be used for screening potential neuroprotective agents. It can also be used for studying drug intervention in other central nervous system diseases.

CHAPTER 5

CONCLUSION

In summary, our study has optimised and set up a PC12-cell based *in vitro* stroke model for screening neuroprotective agents at Universiti Malaysia Sarawak. There are very limited optimisation studies done for *in vitro* stroke model. We have optimised several key factors in developing this *in vitro* stroke model. We found out that DMEM high glucose is the most suitable media for PC12 cells (Riken) and NS-1 cells. Optimisation of cell adhesive substrates showed that both PC12 cells (Riken) and NS-1 cells displayed good morphology with collagen IV. PC12 Adh cells could grow well in all culture conditions. These results show the importance of optimizing cell culture conditions for a PC12 cell variant before using it as an *in vitro* cellular model.

In this study, PC12 cells (Riken) and NS-1 cells were successfully grown in a modified serum-free supplemented media. In contrast, PC12 Adh cells were unable to grow in serum-free supplemented media. Our study is the first to demonstrate that NS-1 cells could grow in serum-free supplemented media. Ability to grow in serum-free supplemented media allows the cell variants to be used in other neurobiological, neurotoxicological or drug screening studies that may require a serum-free environment. We further demonstrated differences among the 3 PC12 cell variants in NGF-induced differentiation. NS-1 cells had the highest response followed by PC12 cells (Riken) and PC12 Adh cells. PC12 Adh cells showed a very minimal response to NGF-induced differentiation and further studies are required to elucidate the reason. NS-1 cells were chosen as the PC12 cell variant for the subsequent experiments. Selecting a PC12 cell variant which can differentiate well into

neuron-like cells is necessary for an *in vitro* model used for screening potential neuroprotective agents in treatment of stroke.

This study showed that 3-hour OGD was the optimal duration to induce cell injury in NS-1 cells. It was the shortest duration to achieve maximal caspase 3 and 7 activity while limiting the proportion of dead cells to facilitate recovery of the culture. This *in vitro* model was validated by using a known neuroprotectant, 8-OH-DPAT. Many studies have reported the neuroprotective effects of 8-OH-DPAT but this is the first report of its neuroprotection in a PC12 cell variant, NS-1 cell. The neuroprotective pathway of 8-OH-DPAT following activation of 5HT_{1A} receptor on PC12 cells is a topic for further studies.

Our data on the optimisation of cell culture conditions, PC12 cell variants and durations of OGD would be useful for investigators planning to develop a PC12 cell-based *in vitro* stroke model. To conclude, the present study has set up an optimal PC12 cell-based *in vitro* stroke model for screening potential neuroprotective agents.

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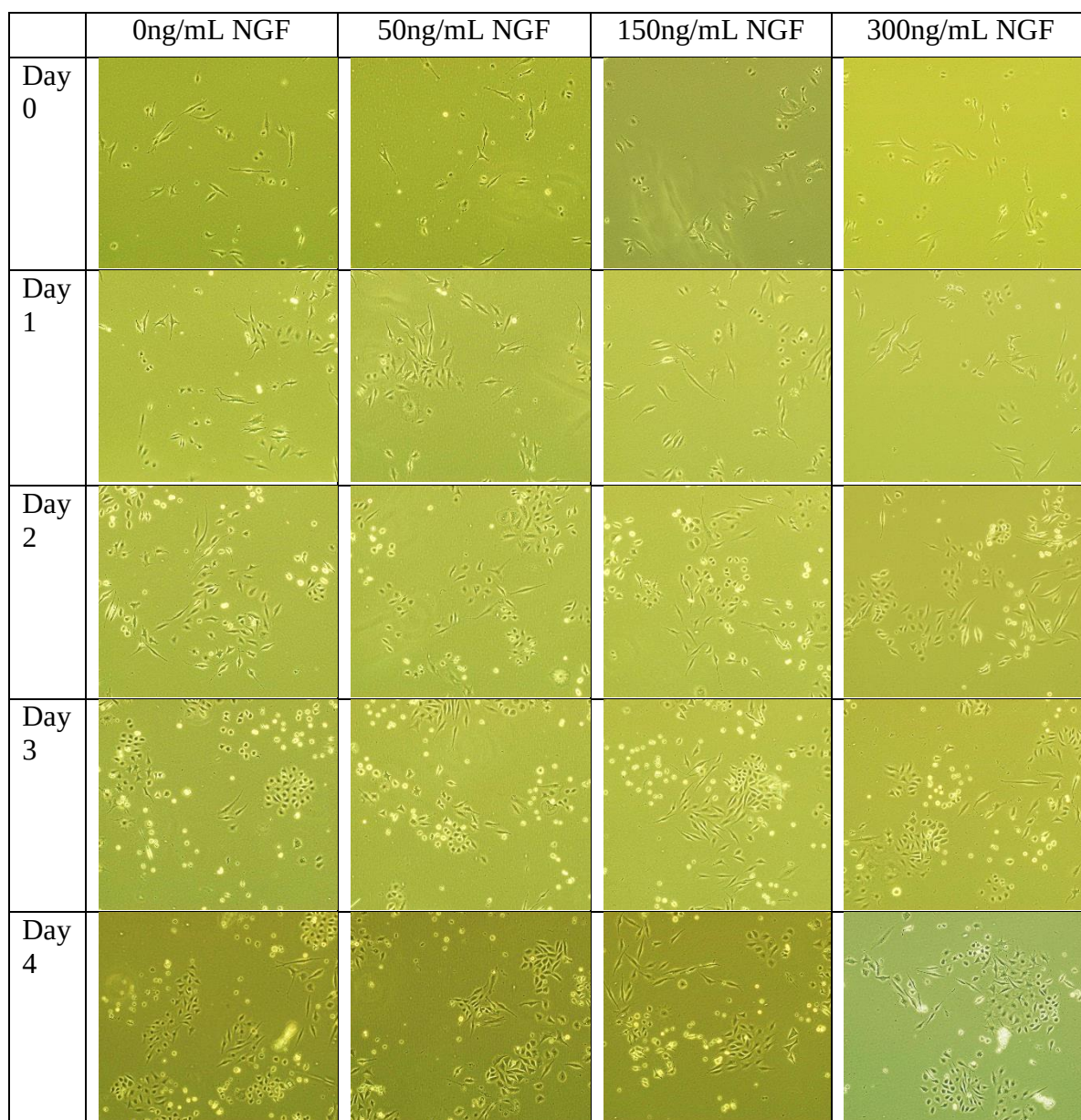
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APPENDICES

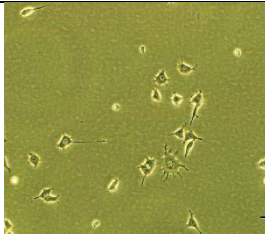
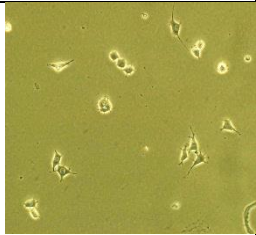
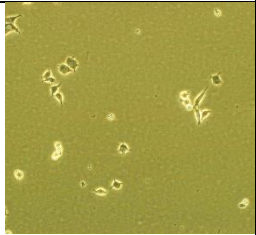
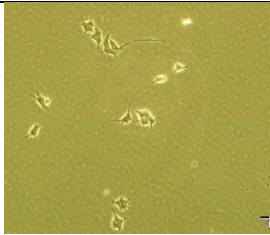
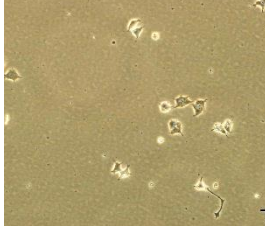
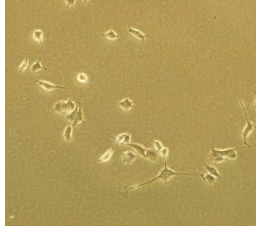
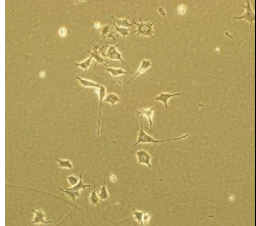
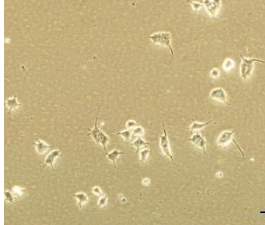
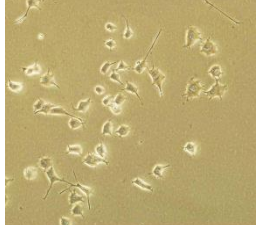
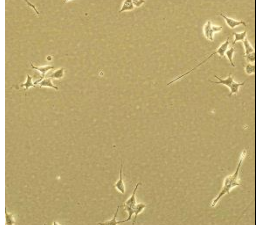
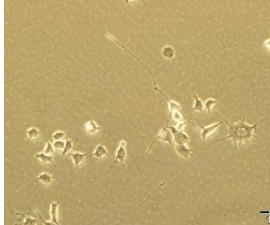
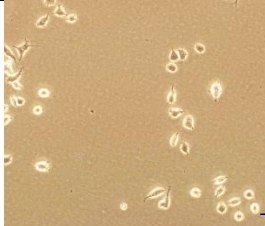
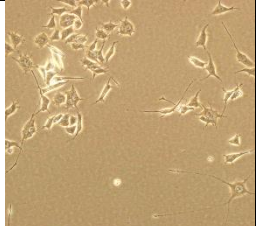
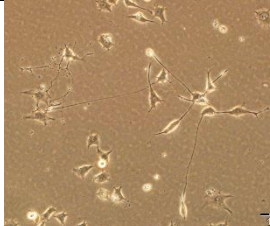
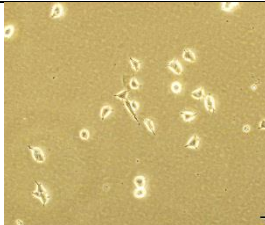
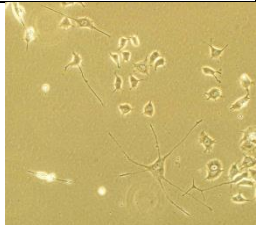
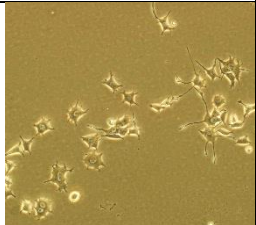
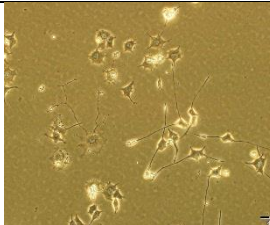
Appendix A: Representative Phase Contrast Micrographs of NGF-induced differentiation from Day 1 to Day 4 of 3 PC12 cell variants.

Appendix A1-A3 shows the representative phase contrast micrographs of NGF-induced differentiation from day 1 to day 4 of PC12 Adh cells (A1), PC12 cells (Riken) (A2) and NS-1 cells (A3) with the concentration ranging 0ng/mL to 300ng/mL).

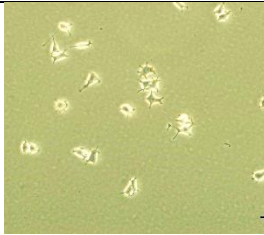
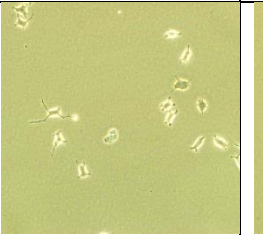
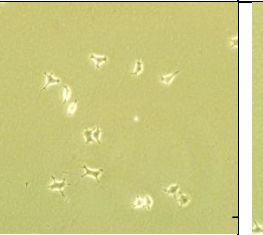
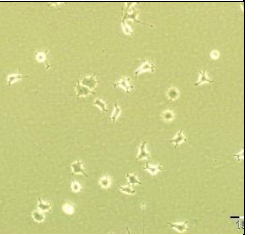
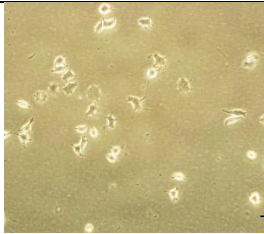
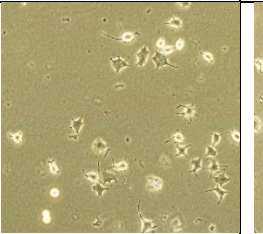
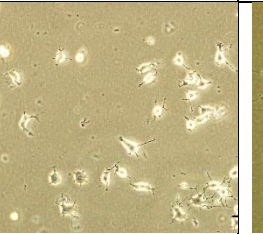
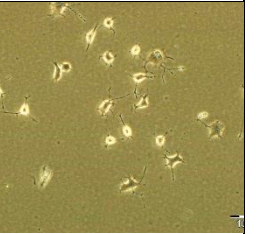
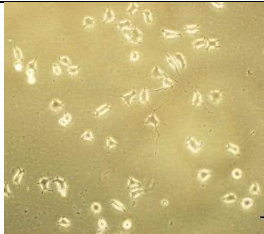
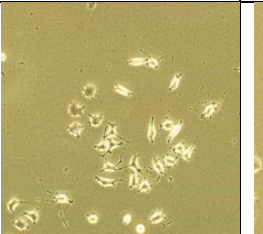
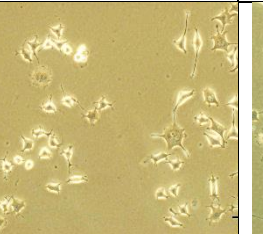
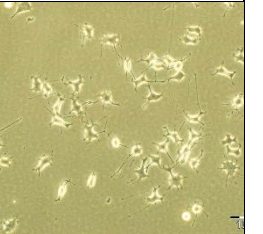
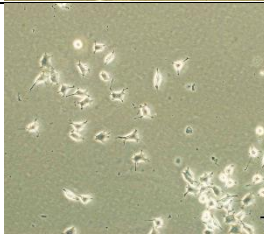
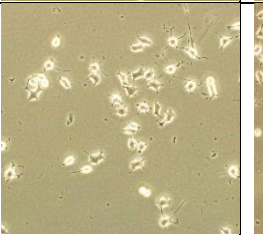
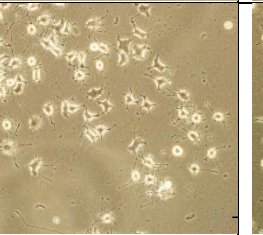
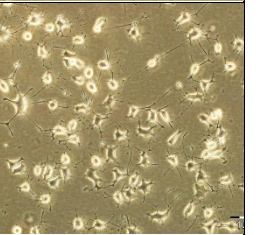
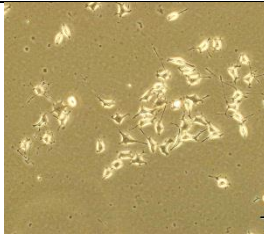
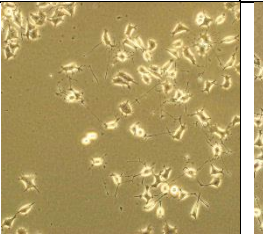
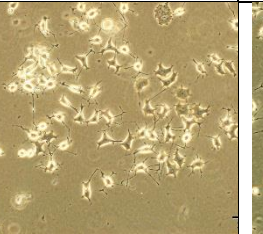
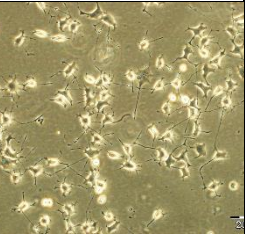
Appendix A1: PC12 Adh Cells (Magnification 100x)



Appendix A2: PC12 cells (Riken) (Magnification 200x)

	0ng/mL NGF	50ng/mL NGF	150ng/mL NGF	300ng/mL NGF
Day 0				
Day 1				
Day 2				
Day 3				
Day 4				

Appendix A3: NS-1 cells (Magnification 200x)

	0ng/mL NGF	50ng/mL NGF	150ng/mL NGF	300ng/mL NGF
Day 0				
Day 1				
Day 2				
Day 3				
Day 4				

Appendix B: Percentage of Neurite-bearing Cells of Three PC12 Cells Variants after 4 Days of NGF-induced Differentiation with Different Concentrations of NGF.

Appendix B shows the percentage of neurite-bearing cells of three PC12 cells variants after 4 days of NGF-induced differentiation with different concentrations of NGF. (Data shown is the mean values $\pm$ SD of 3 independent observations).

Cell variants	Percentage of neurite-bearing cells (%)			
	0 ng/mL	50 ng/mL	150 ng/mL	300 ng/mL
PC12 Adh	6.1 $\pm$ 0.2	5.3 $\pm$ 0.1	6.9 $\pm$ 0.8	4.9 $\pm$ 0.8
PC12 (Riken)	1.9 $\pm$ 0.3	17.5 $\pm$ 5.2	34.4 $\pm$ 8.1	36.0 $\pm$ 5.6
NS-1	36.4 $\pm$ 11.9	58.2 $\pm$ 11.1	64.8 $\pm$ 9.3	72.7 $\pm$ 9.1

Appendix C: Optimisation of NS-1 Cell Density for Caspase 3/7 in White Wall Clear Bottom 96-well Plate.

Appendix C1 shows the procedure of optimisation of NS-1 cell density for caspase 3/7 assay in a white wall clear bottom 96-well plate from day 1 to day 3. Appendix C2 shows the result of the procedure and the cell confluency daily after seeding with different cell density.

Appendix C1: Procedure of Optimisation of NS-1 Cell Density for Caspase 3/7 in White Wall Clear Bottom 96-well Plate.

Day	Procedure
0	Cell seeding into white wall clear bottom 96-well plate. Different cell density was used.
1	Observe the cell confluency for different number of cell seeding and change media to 2% HS DMEM high glucose and add 150ng/mL NGF to mimic differentiation
2	Observe the cell confluency
3	Observe cell confluency

Appendix C2: Confluency of NS-1 cells in from Day 1 to Day 3 after Seeding with Different Seeding Density White Wall Clear Bottom 96-well Plate.

Seeding density	Cell confluency		
	Day 1	Day 2	Day 3
2k	15%	15%	20%
4k	30%	40-50%	60%
6k	40%	60%	70%
8k	70%	80%	90%
12k	80-90%	90%	100%