THE MOLECULAR, CELLULAR AND FUNCTIONAL CHANGES IN THE TREATMENT OF RAT SPINAL CORD INJURY WITH NEURO-PROGENITOR CELLS DERIVED FROM BONE MARROW MESENCHYMAL STEM CELLS

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UNIVERSITI SAINS MALAYSIA

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by

PUTRI NUR HIDAYAH AL-ZIKRI BINTI MOHAMAD AKIL

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

®	Registered trademark
™	Trademark
%	Percent
°C	Degree Celsius
kg	Kilogram
g	Gram
mg	Milligram
µg	Microgram
ng	Nanogram
L	Liter
mL	Milliliter
µL	Microliter
cm	Centimeter
µm	Micrometer
M	Molar
mM	Millimole
ANOVA	Analysis of variance
ASC	Adult stem cell
BBB	Basso, Beatie, and Bresnahan
BDNF	Brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BM	Bone marrow
BM-MSC	Bone marrow derived mesenchymal stem cell
BSA	Bovine serum albumin

CD	Cluster of differentiation
cDNA	Complimentary DNA
CFU-F	Colony Forming Unit Fibroblast
CNS	Central nervous system
Cy3	Indocarbocyanine
CNTF	Ciliary neurotrophic factor
CO_2	Carbon dioxide
DAPI	4′,6-diamidino-2-phenylindole DNA stain
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced Chemiluminescent
ESC	Embryonic stem cell
EGF	Epidermal growth factor
et al	et alii-and others
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial-derived neurotrophic factor
HRP	Horse Radish Peroxide
FBS	Fetal bovine serum
IGF-1	Insulin growth factor 1
ISCT	International Society for Cellular Therapy
LIF	Leukemia inhibitory factor
MSC	Mesenchymal stem cell
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

NGF	Nerve growth factor
NPC	Neural progenitor-like cell
NT-3	Neurotrophin 3
OP	Optical density
PEG	Poly (ethylene glycol)
pHEMA	Poly (2-hydroxyethyl methacrylate)
pHPMA	Poly (hydroxypropyl methacrylate)
PLA	Poly (lactic acid)
PLGA	Poly (lactic-co-glycolic acid)
PBS	Phosphate buffer saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidium Iodide
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RPM	Revolution per minute
SD	Standard deviation
SD	Sprague-Dawley
SDS	Sodium dodecyl sulphate

PERUBAHAN MOLEKULAR, SELULAR DAN FUNGSI DI DALAM RAWATAN KECEDERAAN KORDA SPINAL TIKUS DENGAN SEL NEURO-PROGENITOR DARIPADA SEL STEM MESENKIMA SUMSUM TULANG

ABSTRAK

Kebelakangan ini, terdapat peningkatan minat terhadap pembedahan sel stem mesenchymal (MSCs) kepada keturunan saraf di bawah pengaruh spesifik parakrin. Untuk menguraikan peranan dan kesan faktor parakrin, kami telah membahagikan MSC kepada beberapa jenis sel saraf (NPCs) seperti menggunakan faktor-1 pertumbuhan insulin (IGF-1) dan faktor parakrin asas; faktor pertumbuhan epidermis (EGF) dan faktor pertumbuhan fibroblas asas (BFGF). NPCs yang telah dibezakan dinilai untuk pemulihan fungsi pada saraf tunjang. Pelbagai strategi telah berjaya meningkatkan pemulihan fungsi selepas berlakunya kecederaan saraf tunjang (SCI) tetapi strategi yang optimum masih belum ditemui. Oleh itu, matlamat kajian ini adalah untuk menghasilkan sel stem mesenchymal (MSCs) yang dibahagikan kepada sel-sel saraf (NPCs) selepas kecederaan dan pemindahan sel ke dalam saraf tulang belakang tikus Sprague Dawley untuk menilai kesannya terhadap pemulihan fungsi, histologi, ekspresi gen, dan kajian proteomik. BM-MSCs yang dibahagikan kepada NPCs terdiri daripada 3 kumpulan; Kumpulan A (tanpa faktor pertumbuhan), Kumpulan B (EGF + BFGF), dan Kumpulan C (EGF + BFGF + IGF-1). NPCs (*neurospheres-like cells*) yang terbentuk daripada MSC telah dianalisis untuk pertumbuhan dan kematian sel dengan menggunakan asai MTS dan Annexin V. Dalam kajian vivo, tikus SCI telah dibahagikan kepada tiga kumpulan; Kumpulan 1 (cedera + kenderaan-kolagen), Kumpulan 2 (cedera + kenderaan-kolagen + NPC [bFGF & EGF]) dan Kumpulan 3

(cedera + kenderaan-kolagen + NPC [bFGF, EGF & IGF-1]). Untuk menilai keberkesanan rawatan dan peningkatan fungsi pada T10 tikus SCI, kajian tingkah laku lokomotor dan fungsi deria telah dijalankan. Kesan terhadap tingkah laku tikus dalam kajian vivo adalah faktor yang paling penting untuk menilai keberkesanan rawatan dan peningkatan dalam pemulihan fungsi. Fungsi lokomotor diuji dengan menggunakan penilaian *Modified Open-field Gait* menggunakan skala perkadaran Basso, Beatie, dan Bresnahan (BBB). Koordinasi dan keseimbangan motor ditentukan menggunakan ujian palang kayu. Sementara itu, fungsi deria ditentukan oleh ujian Elektronik Von Frey. Kajian diteruskan dengan penilaian histologi, ekspresi gen dan proteomik selepas siasatan SCI untuk membantu menjelaskan mekanisme berkemungkinana yang membawa kepada pemulihan fungsi seperti yang diperhatikan. Pengasingan BM-MSCs menunjukkan ekspresi positif untuk penanda Vimentin, Nestin, Fibronectin, CD90, dan CD44 manakala ekspresi negatif untuk penanda CD11b. Di antara kumpulan yang dirawat dengan faktor pertumbuhan, pembahagian NPCs yang diperoleh dari BM-MSC yang dirawat dengan IGF-1 menunjukkan percambahan sel tertinggi dan kematian sel terendah. IGF-1 mempunyai peranan penting dalam pembezaan BM-MSCs kepada keturunan saraf dengan meningkatkan percambahan dan mengurangkan kematian sel dalam NPC. Dalam kajian vivo, hasil ujian lapangan terbuka menggunakan skala penilaian BBB menunjukkan bahawa skor purata masa adalah lebih tinggi dalam Kumpulan 3 berbanding Kumpulan 1 dan 2. Perbezaan antara Kumpulan 3 dan kumpulan kawalan adalah signifikan secara statistik pada p <0.05. Sementara itu, terdapat tindak balas positif dalam ujian Von Frey untuk Kumpulan 3 berbanding dengan kumpulan kawalan. Keputusan H&E selepas kajian tingkah laku menunjukkan bahawa Kumpulan 1 memperlihatkan corak yang jelas terhadap kecederaan melalui pengosongan sel pada bahagian jirim putih sebagai tanda nekrosis. Kumpulan 2 dan 3

mempunyai kesan luka yang sangat terbatas yang hampir tidak melintangi kord dan profil kerosakan tisu adalah jauh lebih baik. Walau bagaimanapun, terdapat pembentukan sista yang jelas kelihatan dalam Kumpulan 2. Hasil pewarnaan IHC menunjukkan bahawa semua antibodi jelas kelihatan dalam semua kumpulan termasuk GFAP (untuk *astrocytes*), APC (untuk *oligodendrocyte*), SOX2 (untuk *stemness*), dan MBP (untuk *myelination*) kecuali antibodi Nestin (untuk sel pendahulu neural) selepas pemindahan dijalankan. Keputusan tahap ekspresi gen yang menggunakan perwakilan gen yang terlibat diukur dalam penghasilan atau pembahagian saraf (gen Beta III Tubulin, SOX2, MAP2, Nestin, GFAP, Olig2, dan MBP) dan angiogenesis (gen Ang-1 dan Ang-2). Di peringkat protein, hasil kajian menunjukkan kehadiran penanda saraf yang tidak matang seperti antibodi Beta III Tubulin dan SOX2; dan penanda saraf matang seperti antibodi MAP2 selepas pemulihan SCI, kecuali Nestin. Selain itu, penanda astrocyte (GFAP), penanda oligodendrocyte (APC), dan penanda myelin (MBP) juga dibuktikan dengan kehadiran jalur. Kesimpulannya, transplantasi NPC yang berasal dari MSC pada kecederaan hemiseksi tulang tikus Sprague Dawley mempunyai kesan ke atas pemulihan fungsi termasuk histologi, ekspresi gen, dan tahap proteomik. Oleh itu, maklumat ini akan memberi manfaat untuk meningkatkan terapi berasaskan sel dalam bidang perubatan regeneratif.

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ABSTRACT

Recently, there has been growing interest in the differentiation of mesenchymal stem cells (MSCs) into neural lineages under the specific influence of paracrine factors. To unravel the roles and effects of paracrine factors, we have differentiated BM-MSCs into neural progenitor-like cells (NPCs) using insulin-like growth factor 1 (IGF-1) and standard paracrine factors; epidermal growth factor (EGF) and basic fibroblast growth factor (BFGF). The differentiated NPCs were evaluated for functional recovery in the spinal cord. Various strategies have managed to improve functional recovery after spinal cord injury (SCI) induction but an optimal strategy has not been developed yet. Thus, this study aims to graft Bone Marrow Mesenchymal Stem Cells (BM-MSCs)-derived neural progenitor-like cells (NPCs) after a spinal cord hemisection injury and transplantation into Sprague Dawley rats to assess their effects on functional recovery, histology, gene expression, and proteomic study. BM-MSCs were differentiated into NPCs in three groups; Group A (without growth factor), Group B (EGF + BFGF), and Group C (EGF + BFGF + IGF-1). The BM-MSCs-derived NPCs (neurospheres-like cells) formed were analyzed for cell proliferation and apoptosis using MTS and Annexin V assay, respectively. In *in vivo* study, rats SCI were divided into three groups; Group 1 (injured + vehicle-collagen), Group 2 (injured + vehicle-collagen + NPCs [bFGF & EGF]-treated) and Group 3 (injured + vehicle-collagen + NPCs [bFGF, EGF & IGF-1]-treated). In order to evaluate the treatment efficacy and functional

improvement on T10 rat SCI, behavioural studies on locomotor and sensory function were conducted. The behavioural outcomes in *in vivo* study is the most important factor for evaluating the treatment efficacy and the improvement of functional recovery. The locomotor function was tested by utilizing the Modified Open-field gait assessment using Basso, Beatie, and Bresnahan (BBB) rating scale. Motor coordination and balance were determined using a wooden beam test. Meanwhile, the sensory function was determined by Electronic Von Frey test. The investigation was followed by histology, gene expression and proteomic evaluation after the SCI investigation to help elucidate possible mechanisms leading to the observed functional recovery. Isolation of BM-MSCs showed positive expression on Vimentin, Nestin, Fibronectin, CD90, and CD44 markers whereas negative expression was recorded for CD11b marker. Among the groups treated with growth factors, differentiation of MSCs-derived NPCs treated with IGF-1 showed the highest cell proliferation and the lowest inhibited apoptosis. IGF-1 has a crucial role in the differentiation of BM-MSCs into neural lineage by enhancing the proliferation and reducing the apoptosis in the NPCs. In the *in vivo* study, the open field test using BBB rating scale results exhibited that the mean scores overtime was higher in Group 3 as compared to Group 1 and 2. The difference between Group 3 and the control group was statistically significant at $p<0.05$. Meanwhile, there was a positive response in the Von Frey test for Group 3 as compared to the control group. H&E results after the behavioural study indicated that Group 1 exhibited a clear trend towards injury with vacuolation of cells within the white matter as well as areas of necrosis. Group 2 and 3 had very limited residual lesions and the profile of tissue damage showed considerable improvements. However, there was a cyst formation observed in Group 2. IHC staining results demonstrated that all groups expressed all antibodies including GFAP (for astrocytes), APC (for oligodendrocyte), SOX2 (for stemness), and MBP (for

myelination) antibodies except Nestin (for neural precursor cell) antibody, 4 weeks after transplantation. The results at gene expression level using representative genes involved were upregulated in neural development or differentiation (Beta III Tubulin, SOX2, MAP2, Nestin, GFAP, Olig2, and MBP genes) and angiogenesis (Ang-1 and Ang-2). In the protein level, bands were evident for immature neuronal markers such as Beta III-tubulin and SOX2 antibodies; and mature neuronal markers such as MAP2 antibody after SCI recovery, except for Nestin. Besides, bands were also present for astrocyte marker (GFAP), oligodendrocyte marker (APC), and myelin marker (MBP). As a conclusion, BM-MSCs-derived NPCs transplantation on spinal cord hemisection injury of Sprague Dawley rats demonstrated positive effects on functional recovery including histology, gene expression, and proteomic levels. Therefore, this information will be beneficial for improving cell-based therapy in regenerative medicine.

CHAPTER 1

INTRODUCTION

1.1 Background

Spinal cord injury (SCI) is one of the most commonest debilitating nervous system disorders (Engkasan et al., 2017). It causes interruption of physiological contact between the brain and the body due to neuronal damage. Once SCI occurs, it will lead to pathological conditions which affects both sensory and motor destruction and finally results of the loss of voluntary movements and sensation below the damaged plane (Hosseini et al., 2018, Tran and Silver, 2015).

About 250 000 and 500 000 individuals suffer from SCI worldwide every year, according to the World Health Organization (2019). Most of the causes of SCI are preventable including motor vehicle accident followed by falls, injuries at work and sport and recreation (Mehdar et al., 2019, Badhiwala et al., 2019, Nas et al., 2015). In Malaysia, there is a lack of data regarding the epidemiology of SCI. In the study reported by Ibrahim et al. (2013), out of 292 patients in the study, 224 (77 %) were males with mean age was 39 years, ranging from 2 to 82 years. Motor vehicle collisions have been reported as the primary cause of SCIs (Ibrahim et al., 2013, Engkasan et al., 2017).

Owing to the lack of treatment options, repairing of SCI remains a clinical challenge (Venkatesh et al., 2019, Griffin and Bradke, 2020). Various treatments are available for SCI patients including operative decompression for any residual spinal cord compression, stabilization (Wilson et al., 2017), reconstruction of unstable injuries (Danison et al., 2017), secondary complications management and rehabilitation (Nas et al., 2015, Sezer et al., 2015). Although medical and surgical services has improved the

overall performance of SCI patients, there is currently no effective treatment for severe neurological deficits after SCI. But researchers are actively working on new approach, including medicines and prostheses that can facilitate the regeneration of nerve cells or enhance the function of nerves. Drugs like methylprednisolone are no longer used due to its limited effectiveness and severe side effect (Benzel, 2018).

With the recent advances in regenerative medicine, stem cells transplantation is a promising SCI treatment option (Gazdic et al., 2018, Gao et al., 2020, Goel and Spine, 2016). Stem cell-based therapy provides highly attractive potential for spinal cord repair mechanisms such as spared axons remyelination, inflammation reduction, neuronal circuitry restoration, damaged neuronal and glial replacement as well as angiogenesis promotion (Shao et al., 2019, Griffin and Bradke, 2020, Assunção-Silva et al., 2015, Shi et al., 2017). Even though this progress is encouraging, selecting and sourcing cells for transplantation remains an area for further investigation since cells from various sources have been used including mesenchymal stem cells (MSCs) derived from bone marrow (BM), embryonic stem cells (ESCs), neural stem cells (NPCs), and their derivatives (Quertainmont et al., 2012, Kerr et al., 2010, Fandel et al., 2016, Hosseini et al., 2016). More recently, induced pluripotent stem cells (iPSCs) and their derivative, neuroepithelial-like cells and neural precursor cells, have been successfully used in animal models (Khazaei et al., 2017, Csobonyeiova et al., 2019, Aly, 2020). However, tumorigenic differentiation and genetic and epigenetic defects are associated with the use of iPSCs and their derivative cells (Fujimoto et al., 2012, Amemori et al., 2015, Moradi et al., 2019).

Different form of cells have been transplanted into damages spinal cord, including neural stem/progenitor cells (NSPCs) (Itzhak et al., 2020, Liu et al., 2020a, Sankavaram et al., 2019), Schwann cells (Bunge, 2016, Kanno et al., 2015, Marquardt

et al., 2020), olfactory ensheathing glia (Yao et al., 2018, Anna et al., 2017, Reshamwala et al., 2019), oligodendrocyte progenitor cells (Yang et al., 2018, Lee et al., 2018, Fu et al., 2018), skin-derived precursors (Dai et al., 2018, May et al., 2018, Assinck et al., 2020) and mesenchymal stem cells (Hakim et al., 2019, Cofano et al., 2019, Liau et al., 2020), but no single treatment has been proven successful in treating SCI (Hosseini et al., 2016, Yamazaki et al., 2020, Gao et al., 2020).

Recent studies have also reported that NPCs derived from MSCs have the ability to produce neuronal induction which trans-differentiates MSCs into NPCs with high cell proliferation and survivability with the combination of several growth factors; epidermal growth factor (EGF), basic fibroblast growth factor (bFGf), and insulin growth factor 1 (IGF-1) (Huat et al., 2014, Youssef et al., 2017, Peng et al., 2019). Thus, it would be beneficial to improve the stem cell-based treatment for neurodegenerative disorder like SCI.

The present study was designed to transplant MSCs-derived NPCs (Figure 1.1) in combination with growth factors in order to treat SCI. We hypothesized that the serial combination of all growth factors; bFGF, EGF and IGF-1 would be more effective than when treated with standard bFGF and EGF alone in SCI rat model.

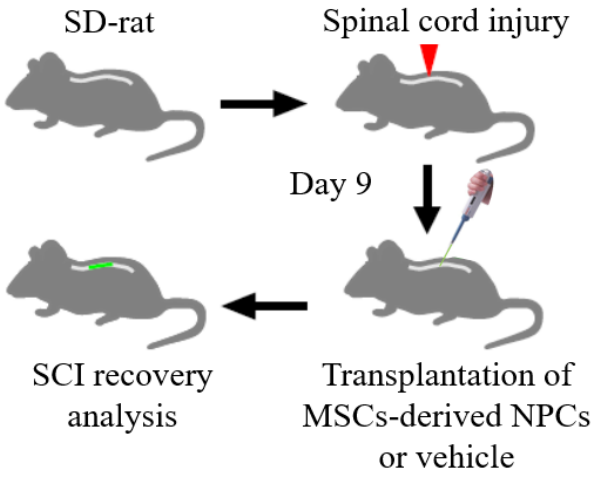


Figure 1.1 Transplantation of BM-MSCs-derived NPCs on SCI rat model.

1.2 Research Objectives

1.2.1 General Objective

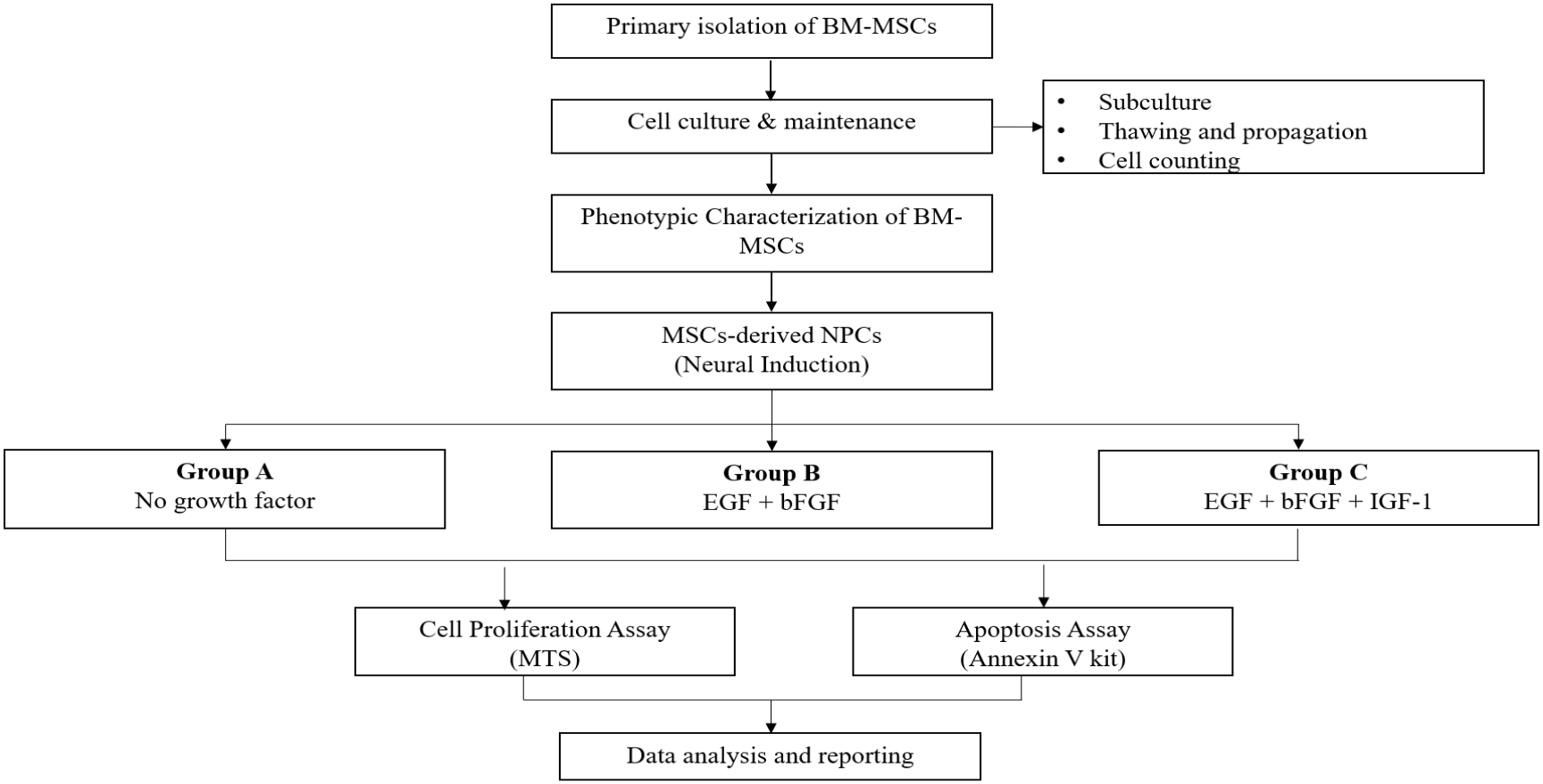
This study was carried out to investigate the ability of BM-MSCs-derived NPCs in the changes of molecular, cellular and functional treatment of rat spinal cord injury.

1.2.2 Specific Objectives

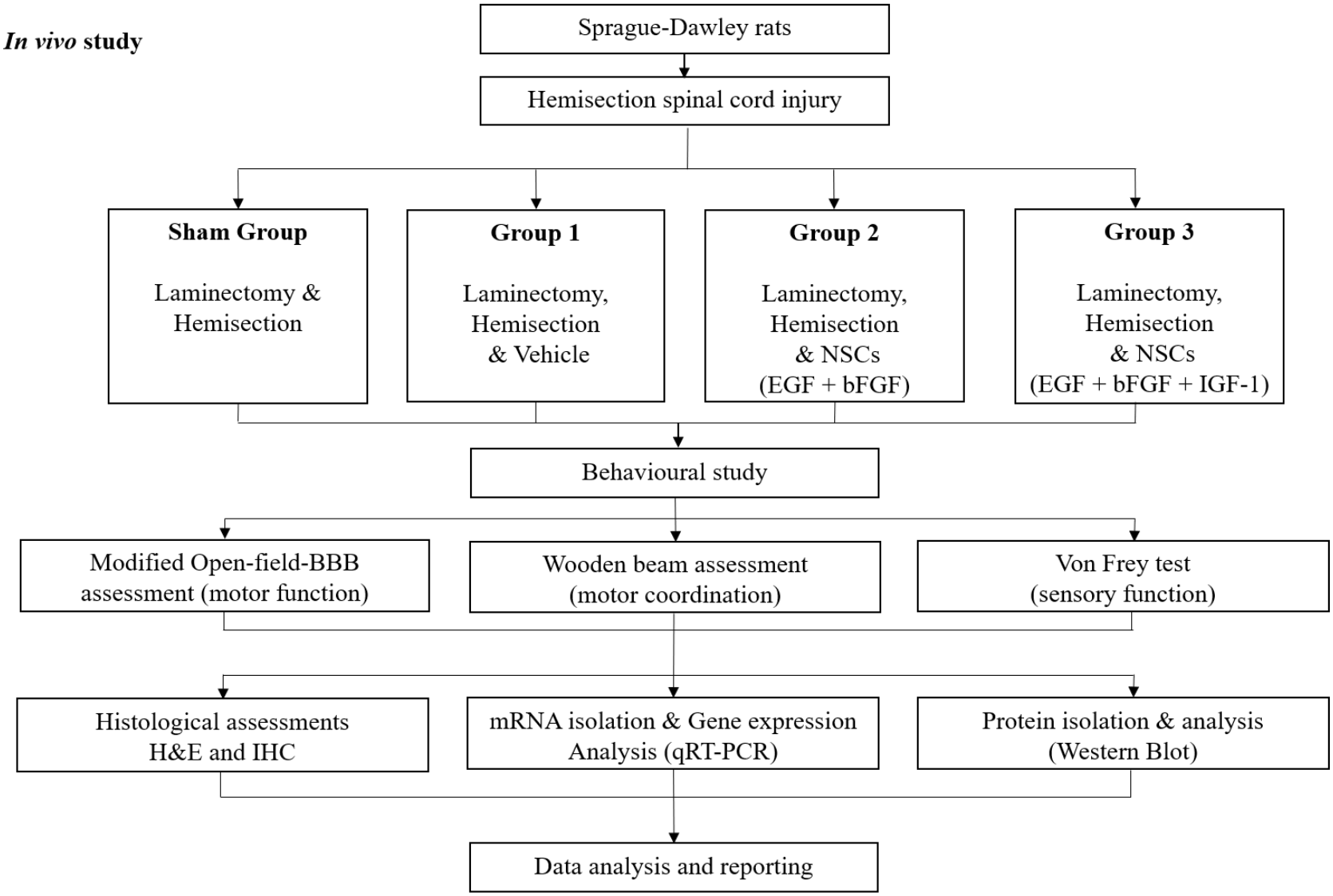
1. To investigate the best combination of growth factors in order to enhance cellular proliferation and survivability of MSCs-derived NPCs *in vitro*.
2. To observe and evaluate the effects of differentiated NPCs from two different groups of growth factors' combination plus scaffold and scaffold alone (vehicle group) towards the locomotor and sensory activities *in vivo*.
3. To observe and analyse the morphology and myelination of the injured spinal cord hemisection area after transplanting with differentiated NPCs in the rat model.
4. To quantify the genes and the intensity of the protein expression involved in the spinal cord injury recovery using quantitative RT-PCR and western blot, respectively, for neuronal development and angiogenesis.
5. To compare and investigate the gene and protein involved in both qRT-PCR and western blot.

1.3 Experimental Design

***In vitro* study**



***In vivo* study**



CHAPTER 2

LITERATURE REVIEW

2.1 Disease of the Nervous System involving the Spinal Cord

Nervous system made up of two parts; peripheral nervous system (PNS) and central nervous system (CNS). The PNS consists of all nerves that branch off from the cord and extend to all areas of the body whereas the CNS consists of the brain and spinal cord. The nervous system transmits signals through internal organs between the brain and the body. Therefore, it will regulate the ability to control all functions of the body including breathe, move, think, see, and more (MedlinePlus, 2016, 2018, Ludwig et al., 2020).

Nervous system's problem can happen slowly and cause a gradual dysfunction (degenerative). Or they can occur unexpectedly and cause life-threatening problem (acute). The symptoms can be extreme or moderate. Some serious diseases, illnesses, and injuries that can cause nervous system problems include: blood supply problems (vascular disorders); injuries (trauma), particularly head and spinal cord injuries; birth problems (congenital); mental wellbeing issues, such as depression or psychosis, anxiety disorders, exposure to toxins such as arsenic or lead, carbon monoxide; problems causing gradual loss of function (degenerative), such as Parkinson's disease, multiple sclerosis (MS), Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Huntington's disease, and peripheral neuropathy (Healthwise, 2019, Zimmermann, 2018).

Nerve diseases include acute injury and chronic disease like neurodegeneration disease which can cause various functional disorders of nervous systems (Tian et al., 2015). Acute nerve injuries, such as traumatic brain injury (TBI), spinal cord injury (SCI), and peripheral nerve injury (PNI), are classified based on the nerve components'

damage, functionality and spontaneous recovery capacity (Tuncel et al., 2011). Nerve disease may affect body activities such as heart function, talking, breathing, balance and movement.

2.2 Human Spinal Cord

The spinal cord and the brain together make up the central nervous system (CNS) which is responsible for receiving information from and sending information to the peripheral nervous system. The spinal cord coordinates the body's movement and sensation and the brain processes and interprets sensory information from the spinal cord. The spinal cord is organized into several segments along its length which are cervical, thoracic, lumbar, sacral and coccyx (Figure 2.1). Nerves from each segment bind to specific regions of the body. The segments in the neck (cervical region) that control signals to the neck, arms, and hands are referred to as C1 through C8. Those in the thoracic or upper back region (T1 through T12) convey signals to the torso and certain parts of the arms. The lumbar (mid-back region) under the ribs (L1 through L5) control signals to the hips and legs. Lastly, the sacral segments (S1 through S5) lie just below the lumbar segments in the mid-back and control signals to the groin, toes, and some parts of the legs (Rea, 2015, Antoine Micheau and Hoa, 2009).

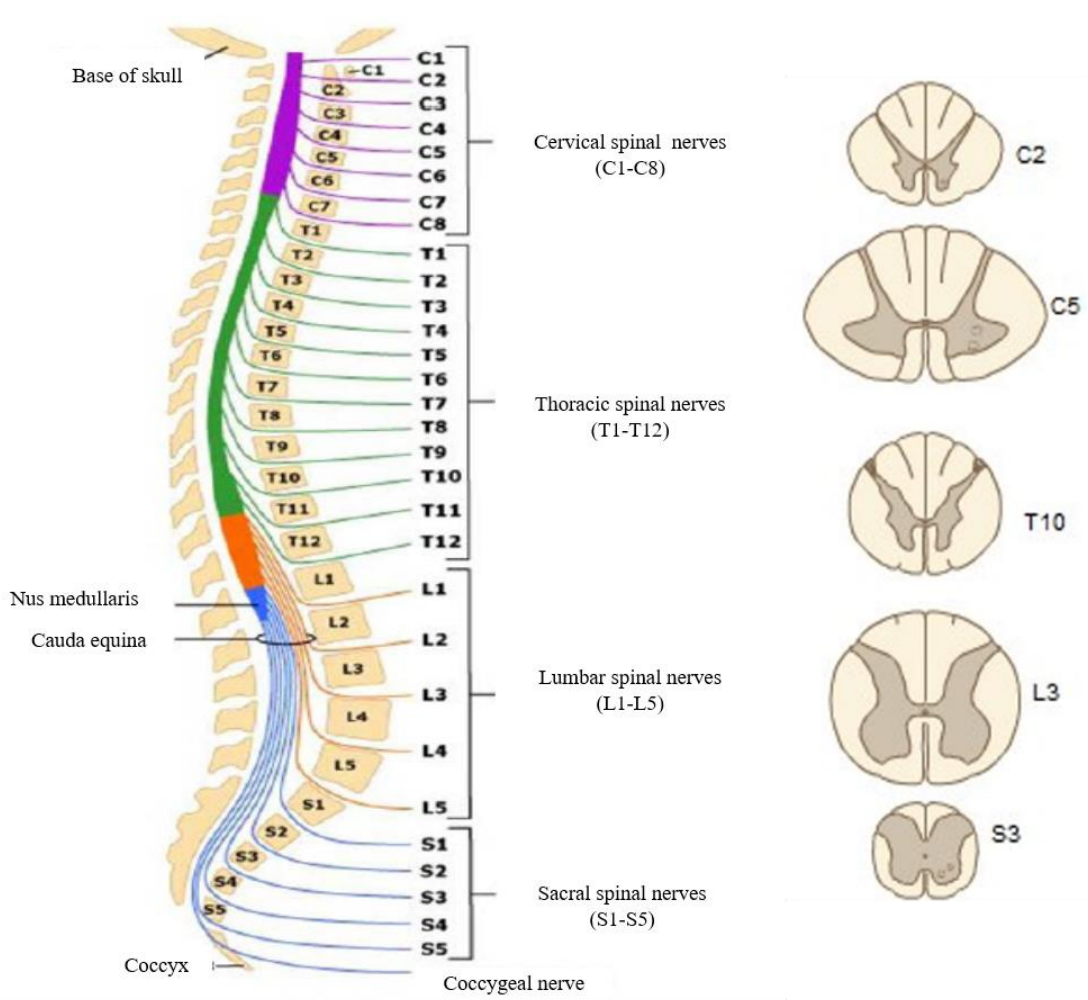


Figure 2.1 Anatomy of the human spinal cord (Dlamini, 2020).

2.3 Rat Spinal Cord

In this study, Sprague-Dawley rats will be used as a spinal cord injury model. The rat spinal cord differs from the human spinal cord, but the behavior responses are relevant to the clinical signs of SCI in human patients. The size and the position of the ascending (sensory) and descending (motor) spinal cord tracts differ between rat and human spinal cord (Figure 2.2). The rat spinal cord is comprised of 34 segments which are 8 cervical, 13 thoracic, 6 lumbar, 4 sacral and 3 coccygeal (Figure 2.3). The effects of spinal cord injury at different segments along the spine reflect this organization (Watson et al., 2009, Alizadeh et al., 2019, Chin, 2018). Therefore, we decided to induce spinal cord hemisection injury at T10, wherewith T10 injury, rats can control their forelimbs and hindlimbs as well as abdominal muscles and trunk. This situation will only cause the hip flexors and legs to be compromised, but not affect the sitting balance (Krishna et al., 2013, Verma et al., 2019). Figure 2.4 shows the section thoracic through the vertebra.

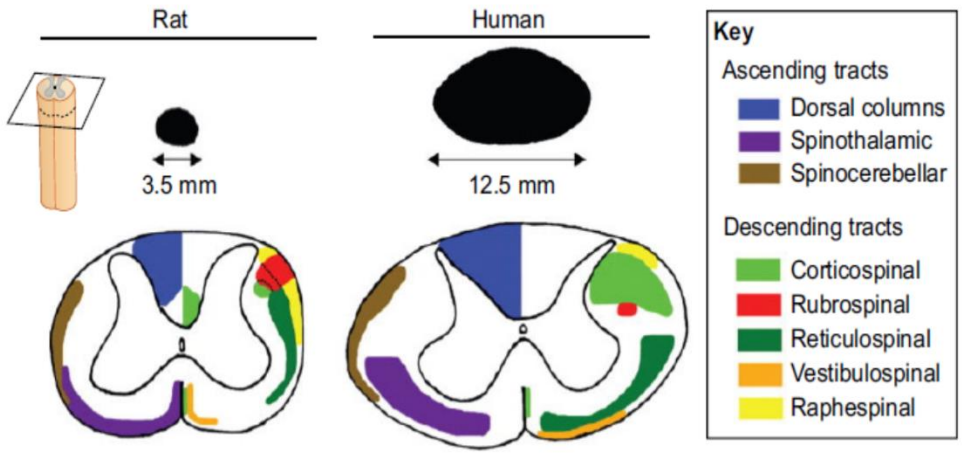


Figure 2.2 Differentiation of the rat and human spinal cord (Kjell and Olson, 2016).

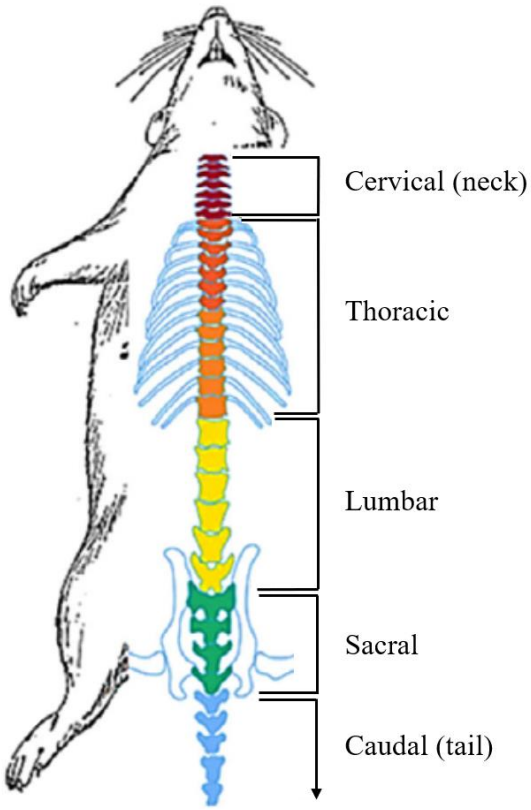


Figure 2.3 Anatomy of the rat spinal cord (Ramotowski et al., 2019)

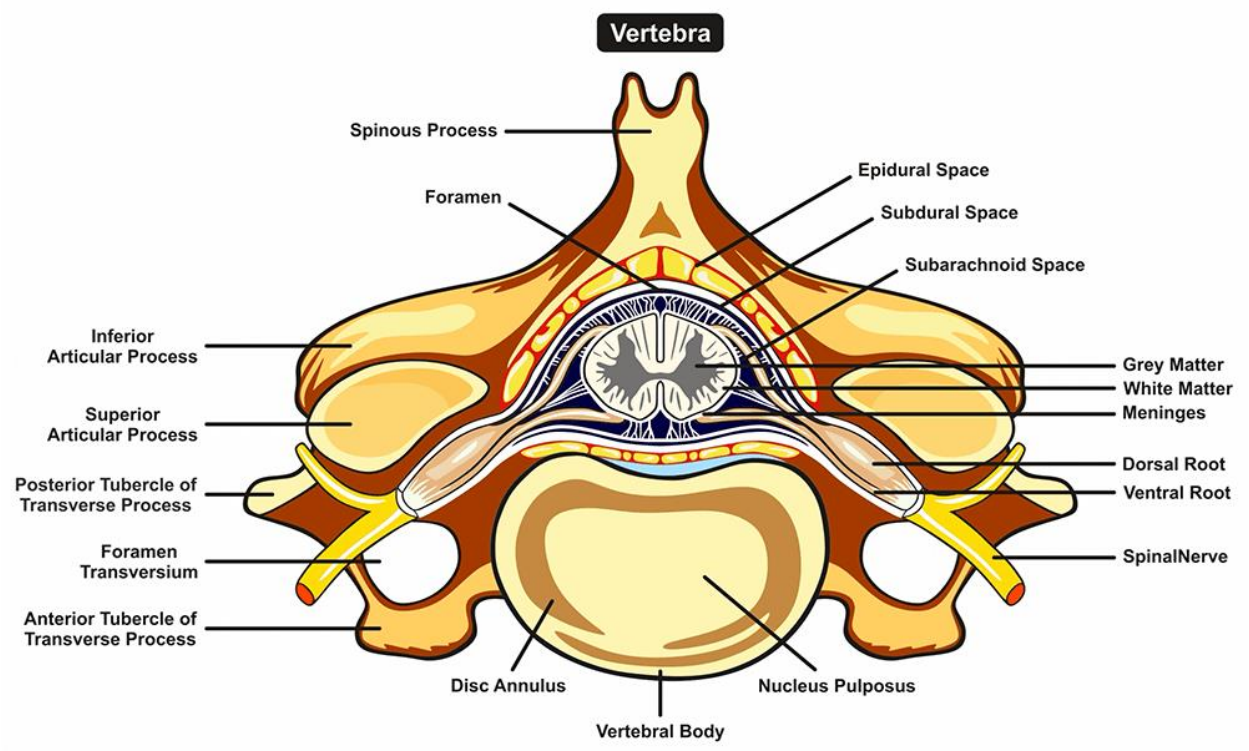


Figure 2.4 Section through thoracic vertebra (Downey, 2018).

2.3.1 Anatomy and Histology of Spinal Cord

Once presented in a cross-section, the spinal cord made up of central gray matter and peripheral white matter. The gray matter has a butterfly shape and is divided into three pairs of horns which are dorsal (posterior), lateral (only present between the first thoracic and second lumbar spinal levels), and ventral (anterior) (Figure 2.5). Sensory neurons join the spinal cord through the dorsal horn, while lateral (autonomic nervous system, sympathetic branch) and ventral (somatic nervous system and autonomic nervous system, parasympathetic branch) horns originate from motor neurons (Tallitsch and Guastaferri, 2009, Khan and Lui, 2020, Nógrádi and Vrbová, 2013, Chawla, 2016). The ventral horn comprises of the cell bodies of the motor neurons that transmit axons to striated muscles through ventral roots of the spinal nerves to terminate on. However, in the white matter, it is split into dorsal, lateral, and ventral columns, with its own axon

tracts.The dorsal columns carry ascending sensory information, the lateral columns comprise of axons moving from the cerebral cortex to contact spinal motor neurons, whereas the ventral columns carry ascending information about pain and temperature, and descending motor information. As a summary of spinal cord organization, neurons and axons that process and relay sensory information are dorsally distributed, preganglionic visceral motor neurons are found in an intermediate/lateral region, and somatic motor neurons and axons are located in the ventral portion of the spinal cord (Purves D et al., 2001, Al-Chalabi and Alsalman, 2019, Ganapathy et al., 2020).

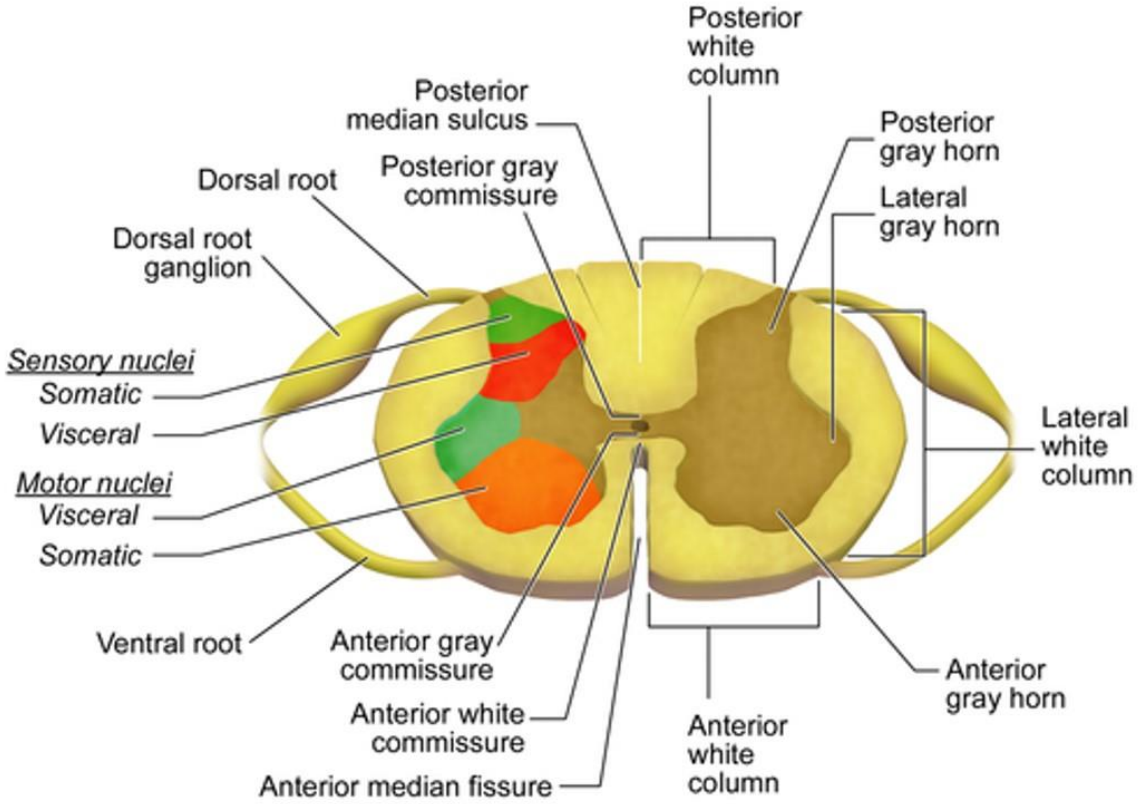


Figure 2.5 Sectional Organization of the Spinal Cord (Ganapathy, 2020).

Cells of the CNS are typically consist of two major categories of cells which are cells of neuroectodermal origin and cells of mesenchymal origin. Cells of neuroectodermal origin divided into four which are neurons, oligodendrocytes, astrocytes, and ependymocytes. Whereas cells of mesenchymal origin consist of meninges, adipose tissue, blood vessels, and microglia (Garman, 2011, Ban and Mladinic, 2020, Hosseini et al., 2020).

Spinal cord in the CNS consists of two kinds of cells which are neurons and glial cells (Figure 2.6). Neurons (nerve cells) are functional units of information and signal processing whereas glial cells provide essential support to neurons. Neurons can be characterised by wide variations in size (either small or wide neurons) and shape. The cell bodies of neurons have multiple dendrites but for rare exceptions, each neuron has only one axon (however, this axon may branch to its cell body at distal points). Axons are specialized for transport, depolarization wave conduction, and synaptic transmission. The large neurons can be characterized by relatively large cell bodies and single prominent nuclei. Glial cells are the most abundant cell types in CNS (brain and spinal cord) which include astrocyte, oligodendrocytes, and microglial cells (Purves D et al., 2001, Domingues et al., 2016, Jäkel and Dimou, 2017, Queensland Brain Institute, 2017). The main function of glial cell is to surround and hold neurons in place, to supply neurons with nutrients and oxygen, to insulate one neuron from another, and to destroy pathogens and remove dead neurons (Purves D et al., 2001). Astrocytes (astroglia) are star-shaped cells which provide mechanical and metabolic support to the neurons (Domingues et al., 2016, Jäkel and Dimou, 2017). They participate in the maintenance of the composition of the extracellular fluid. In gray matter regions, astrocytes are often found to be in close proximity to neurons and anywhere within the neuropil. Astrocytes

also can be identified with pale and small nuclei (Garman, 2011, Şovrea and Boşca, 2013). Oligodendrocytes (oligoglia) form the myelin sheath around some of the CNS's axons, but not all of them. Myelin has important effects on the speed of action potential conduction. Oligodendrocytes are commonly found adjacent to neuron cell bodies inside the gray matter. Therefore, they are also reffered to as satellite cells. Oligodendrocyte can also be identified with small nuclei and surrounded by rings of cytoplasm in gray matter. (Garman, 2011, Domingues et al., 2016). Microglia cells are smaller cells with complex shapes originating from hematopoietic stem cells and some of them derived directly from neural stem cells. Their functions are mainly as scavenger cells which eliminate cellular debris from site of injury or normal cell regeneration (Purves D et al., 2001, Arcuri et al., 2017, Sierra et al., 2019). Figure 2.7 shows the cross-section of neurons and glial cells in the gray and white matter of the spinal cord.

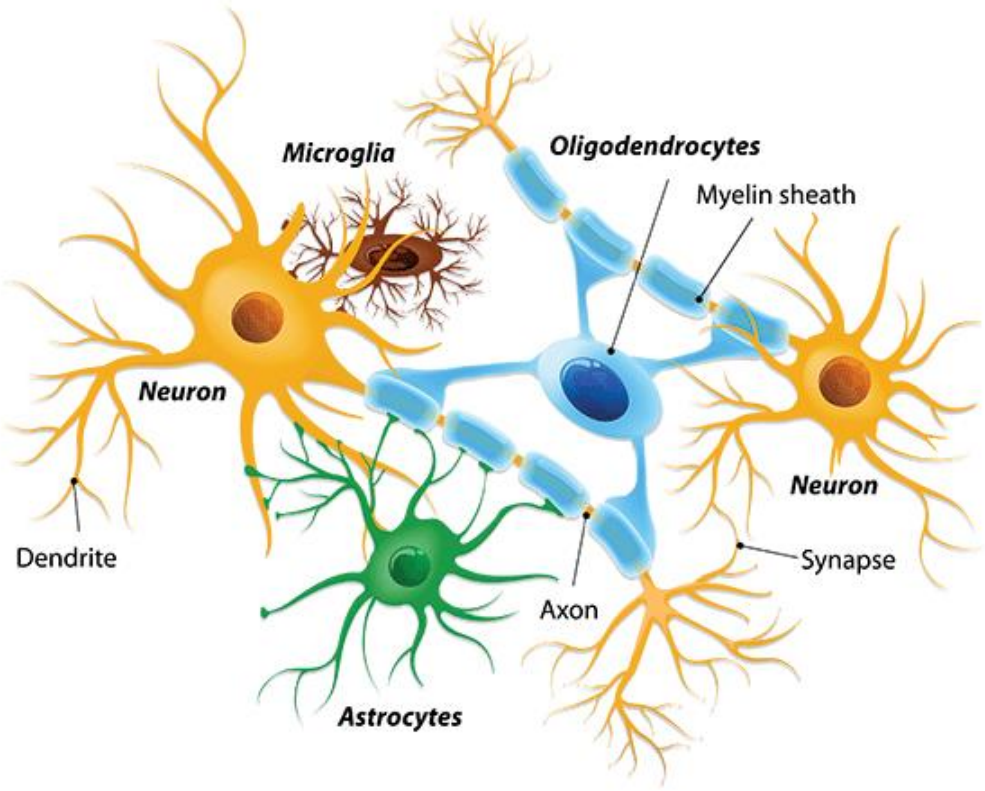


Figure 2.6 Types of glial cells; astrocytes, oligodendrocytes, and microglia, and neuron in the Central Nervous System (Brookshire, 2017).

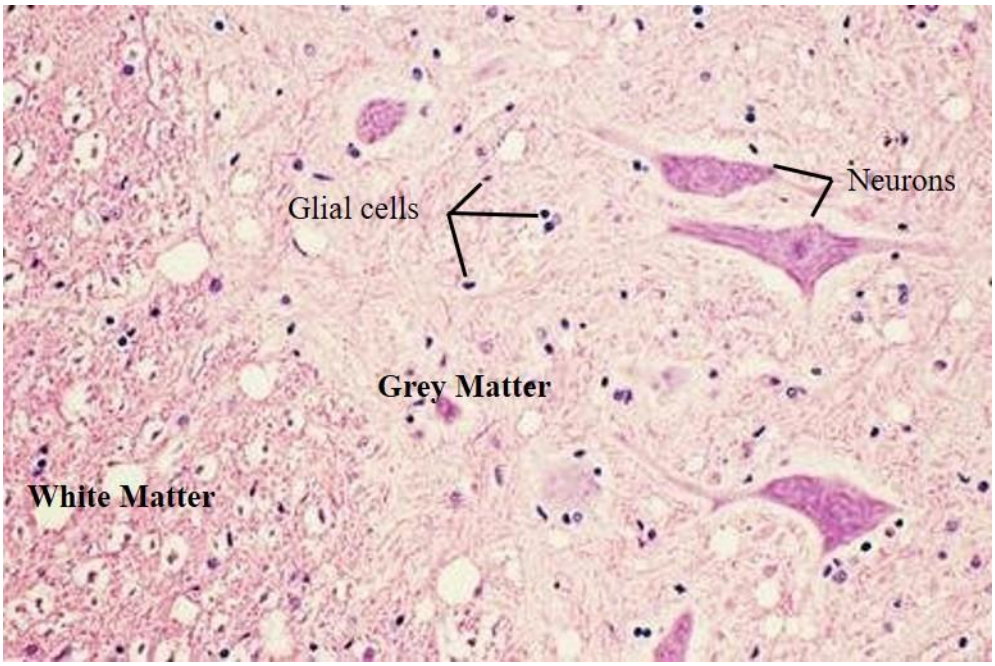


Figure 2.7 Cross-section of neurons and glial cells in the gray and white matter of the spinal cord (Arcuri et al., 2017).

2.3.2 Spinal Cord Injury

Spinal cord injury (SCI) is an injury to the spinal cord due to the damage of axons, neural cell membranes, and rupture of the blood vessels. This injury alters the normal motor, sensory or autonomic function of the cord by affecting the ability of the spinal cord to send and receive signals from the brain to the body's systems that control sensory, motor and autonomic functions below the level of injury (Ribotta et al., 2002, Rubin, 2020).

2.3.2(a) Mechanism of Spinal Cord Injury

Mechanisms of injury in spinal cord models can be categorised as contusion, compression, dislocation, distraction, transection, or chemical (Paterniti et al., 2018, Cheriyan et al., 2014, Pinchi et al., 2019). Contusion, compression, and transection are among the famous models in spinal cord injury. In the contusion model, a transient force is required to displace and injure the spinal cord. In spinal cord contusion models, researchers use devices such as weight-drop, air pressure device, and electromagnetic to induce injury (Verma et al., 2019, Mohammadshirazi et al., 2019). Compression models are identified by compression of the spinal cord for a prolonged period of time (Sun et al., 2017, Su et al., 2015). Transection models involve partial or complete severing of the spinal cord at a particular level. In order to study neuronal regeneration following injury, complete and partial transection models have been utilised extensively. It is because they are beneficial for axonal regeneration evaluation and subsequent functional recovery (Cheriyan et al., 2014, Alizadeh et al., 2019, Lukovic et al., 2015).

2.3.2(b) Partial Transection of Spinal Cord Injury

Partial transection, also known as unilateral transection or hemisection (Figure 2.8) of the spinal cord is the process of selectively lesioning the spinal cord. This model stimulates an injury more likely to be seen clinically than the complete transection (Alizadeh et al., 2019). It also provides for comparison between injured and healthy fibres in the same animal. In addition, hemisection model results in a less severe injury and post-operative animal care is easier as compared to complete transection (Kwon et al., 2002, Alizadeh et al., 2019, Brown and Martinez, 2019).

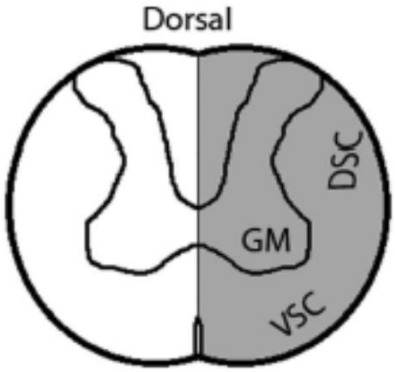


Figure 2.8 Schematic diagram of partial transection of the spinal cord. Gray matter (GM), ventral and dorsal spinal cord (VSC and DSC) (Cheriyan et al., 2014).

2.3.3 Histology of Spinal Cord Injury

SCI results in the death of cells and progressive oligodendrocyte demyelination. Demyelinated axons undergo major changes in physiological and molecular reorganizations that result in degeneration and sensory loss, axonal dysfunction and motor functions (Castejón, 2018, Ohno and Ikenaka, 2019, Alizadeh et al., 2015). In

this situation, endogenous adult precursor cells for oligodendrocyte and neural stem/progenitor cells will lead to the replacement of oligodendrocytes. However, the amount and quality of endogenous remyelination are minimal and substandard. Emerging evidence suggests that multiple factors limit optimal remyelination, including low levels of oligodendrogenesis-promoting factors, cell death among newly generated oligodendrocytes, and poor expression of key regulators that are essential for proper re-construction of a highly organized myelin sheath (Alizadeh et al., 2015, Czepiel et al., 2015, Alizadeh and Karimi-Abdolrezaee, 2016).

2.3.4 Level of Spinal Cord Injury

SCI causes two levels of injuries which are primary and secondary injury (Anwar et al., 2016, Fehlings, 2019, Highsmith, 2018). Primary injury occurs through the direct injury of the axons, neurons, and blood vessels due to the distraction, transection, and even compression of the spinal cord. After that, secondary injury mechanisms begin immediately and cause secondary spinal lesions. Secondary spinal lesions consist of haemorrhages, vasospasm, spinal cord edema and hypoperfusion of the spinal cord. This damage on the spinal cord will continue to progress for several days to weeks and may lead to the death of neurons, demyelination and the interruption of the axonal tracts or axonal loss (Alizadeh et al., 2015, Katoh et al., 2019). The secondary spinal cord injury could be stopped or decreased by early efficient treatment (Lencean and Lencean, 2016). The secondary injury can be divided into acute, sub-acute and chronic phases. The acute phase begins immediately (seconds to minutes) after SCI. It includes vascular damage, neurotransmitter accumulation (excitotoxicity), ionic imbalance, free radical formation, calcium influx, inflammation, edema, lipid

peroxidation, and cell death (Oyinbo, 2011, Alizadeh et al., 2019, Anjum et al., 2020, Naseem and Parvez, 2014). As the injury progresses, the sub-acute injury phase begins (minutes to weeks after SCI). It involves demyelination of surviving axons, apoptosis, axonal die-back, matrix remodelling, Wallerian degeneration, and evolution of a glial scar around the injury site. Further changes occur in the chronic phase (months to years after SCI) including the formation of cystic cavity, maturation of glial scar, and progressive axonal die-back (Oyinbo, 2011, Alizadeh et al., 2019, Tran et al., 2018, Alizadeh et al., 2015).

2.3.5 Biomarkers for Spinal Cord Injury

In traumatic SCI, the mechanical injuries can directly cause the axonal destruction in fibre tracts, destruction of the glial cells and the neurons in gray matter. This destruction may release substances of cellular constituents which can be lesional biomarkers. The lesional biomarkers appear immediately after injury induction and after several hours, reaction biomarkers appear that are involved in the secondary cellular response to injury (Lencean and Lencean, 2016, Albayar et al., 2019).

Biomarkers have the capacity to be sensitive thus an accurate tool to measure neuronal injuries and to predict the evolution of the injury. It also has measurable features that can be used to confirm the presence or to predict the severity of disorders. In this study, several biomarkers were evaluated to detect neuronal injury and angiogenesis which are SOX2, Nestin, Beta III-tubulin, MAP-2, GFAP, APC, Olig-2, MBP, Ang-1, Ang-2, and Tie1.

SOX2 is also known as SRY (Sex determining region-Y)-box 2. It is a persistent marker at all stages of development for multipotential neural stem cells and plays a

crucial role in the maintenance of embryonic and neural stem cells (Cawsey et al., 2015, Ellis et al., 2004, Novak et al., 2019, Zhang and Cui, 2014).

Nestin is a class IV intermediate filament protein which was first described as a marker for neural stem/progenitor cells and expressed in the early stages of development (Matsuda et al., 2013, Cawsey et al., 2015, Xie et al., 2015).

Beta III-tubulin or class III beta -tubulin isotype is usually recognised in developmental neurobiology and stem cell research as a neuronal marker (Dráberová et al., 2008, Mariani et al., 2015, Foudah et al., 2014). Beta III-tubulin is also known as Tuj-1 and its expression particularly correlates with the earliest stages of neuronal differentiation. Therefore, for this reason, beta III-tubulin is used as an indication for neurogenesis, axonal guidance, and maintenance (Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Cho et al., 2020)(Hugo Gene Nomenclature, 2020, Cho et al., 2020).

Microtubule-Associated Protein 2 (MAP-2) is a dendrite-specific protein used as a marker for dendritic injury. MAP-2 functions to stabilize neuronal shape by promoting the synthesis of microtubule and cross-linking with other cytoskeleton components (Yokobori et al., 2015, Morrison et al., 1998, Melková et al., 2019).

Glial Fibrillary Acidic Protein (GFAP) is an intermediate filament protein expressed in glial cells (astrocytes) mainly found in the CNS (Yokobori et al., 2015, Albayar et al., 2019). GFAP is responsible for the formation and development of the cytoskeleton of astroglial cells, and for injury and dysfunction. GFAP is an established

biomarker for traumatic brain injury and promises to be applied similarly in SCI (Hulme et al., 2017, Diaz-Arrastia et al., 2014, Yang and Wang, 2015).

Adenomatous polyposis coli (APC) is specifically labelled mature oligodendrocyte cell bodies in the CNS without labeling myelin. APC is also known as APC clone CC1 (Valério-Gomes et al., 2018, Bin et al., 2016, Behrangi et al., 2020).

Oligodendrocyte transcription factor (Olig-2) is a basic helix-loop-helix (bHLH) transcription factor encoded by the Olig-2 gene. Olig-2 is a well known biomarker for the detection of oligodendrocyte differentiation and replication in early development (Ligon et al., 2004, Dennis et al., 2019, Quintela-López et al., 2019).

Myelin basic protein (MBP) is a protein synthesised by oligodendrocyte and/or Schwann cell. MBP is the main component of the myelin sheath which is wrapped around the axon and as much as one-third of myelin protein. MBP is found abundantly in the white matter where the compact myelin sheath is formed and maintained (Yokobori et al., 2015, Campagnoni, 1988, Stadelmann et al., 2019, Snaidero and Simons, 2014).

Angiopoietin-1 (Ang-1) plays a critical role in regulating angiogenesis and vessel maturation. In order to promote chemotaxis, cell proliferation, cell sprouting, vessel growth and stabilisation, Ang-1 stimulates Tie-2 signalling on endothelial cells (Mueller and Kontos, 2016, Li et al., 2019a, Jeansson et al., 2011).

Angiopoietin-2 (Ang-2) is a secreted protein growth factor that promotes angiogenesis. It binds to the receptor tyrosine kinase, which is expressed on endothelial cells and important for maintaining the integrity of adult blood vessels (Fiedler and Augustin, 2006, Wu et al., 2017, Akwii et al., 2019).