INHIBITION OF NEURONAL NITRIC OXIDE SYNTHASE EXPRESSION AND PREVENTION OF MITOCHONDRIAL DYSFUNCTION AT SPINAL VENTRAL HORN AFTER C7 SPINAL ROOT AVULSION IN RATS WITH TAXOL

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

BDNF	Brain-Derived Neurotrophic Factor
BPI	Brachial Plexus Injury
CcO	Cytochrome c Oxidase
DAB	Diaminobenzidine
DMSO	Dimethyl Sulfoxide
GDNF	Glial Cell-derived Neurotrophic Factor
HCL	Hydrochloric Acid
KH_2PO_4	Monobasic Potassium Phosphate
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
nNOS	Neuronal Nitric Oxide Synthase
NaOH	Sodium Hydroxide
O_2^-	Superoxide Anion
$^{\cdot}OH$	Hydroxyl Radical
$ONOO^-$	Peroxynitrite
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
ROS	Reactive Oxidation Species
SCI	Spinal Cord Injury

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ABSTRACT

Introduction

Functional outcome following surgical repair in brachial plexus avulsion injury remains poor. Spinal motorneuron death after brachial plexus avulsion injury has been identified as the neurobiological barrier to functional restitution. Post injury oxidative stress reaction, for example, up-regulation of neuronal nitric oxide synthase (nNOS), not only cause direct damage to the motoneurons, but lead to mitochondrial dysfunction as well, especially the cytochrome c oxidase (CcO) activity, which serve as the main energy generator for neuronal normal activities. Furthermore, the impaired retrograde axonal transport of neurotrophic factors (which are vital for motoneurons survival) secondary to neurofibrogenesis and mitochondrial dysfunction has retarded the neuronal regeneration process. Taxol, a diterpene alkaloid, has the effect in slowing the neurofibrogenesis by microtubule stabilization and facilitate axonal regeneration in rats. This study was designed to evaluate the neuroprotective effect of intrathecally infused Taxol in the prevention of motoneuron death and mitochondrial dysfunction following brachial plexus avulsion injury.

Material and Method

Sprague-Dawley rats were divided into Treatment and Control groups (each group N=32). Brachial root avulsion injury was induced in each rat. The Treatment group received 5 days intrathecal infusion of Taxol (256ng/day) via a micro infusion pump, whereas the Control group received normal saline. Cervical cord was harvested at survival interval of 1 week, 2 weeks, 4 weeks and 6 weeks (n=8 in each subgroup). Number of surviving motoneurons and nNOS-positive motoneurons at injured

ventral horn were determined with NADPH-d histochemistry with neutral red counterstaining. Mitochondrial function at the injured ventral horn was measured with CcO histochemistry and densitometer. Independent *t*-test was applied to detect differences between the study groups at specific survival interval.

Results

Compared to Control group, the Taxol treated group showed significant reduction in the nNOS expression at 2 weeks, 4 weeks, and 6 weeks, and significantly improved mitochondrial functions at 4 weeks and 6 weeks. The motoneurons survival rate was significantly increased at 2 weeks, 4 weeks, and 6 weeks in Taxol treated rats.

Conclusions

Taxol has the neuroprotective effect to prevent spinal motoneuron degenaration following brachial plexus avulsion injury by inhibiting nNOS expression and preventing mitochondrial dysfunction.

ABSTRAK

Pengenalan

Pemulihan fungsi berikutan pembedahan perbaikan untuk kecederaan avulsi brachial plexus masih kurang memuaskan. Kematian pada motoneuron saraf tunjang selepas kecederaan avulsi brachial plexus telah dikenalpasti sebagai halangan utama dalam pemulihan fungsi. Reaksi tekanan oksidatif selepas kecederaan, sebagai contoh, kenaikan regulasi neuronal nitric oxide synthase (nNOS), bukan sahaja telah merosakkan motoneuron, malah ia juga menyebabkan ketidakfungsian mitokondria, terutama sekali aktiviti cytochrome c oxidase (CcO), yang merupakan penghasil tenaga utama untuk aktiviti-aktiviti normal neuron. Selain itu, kegagalan pengangkutan axonal songsang untuk faktor-faktor neutrofik (yang amat penting untuk kehidupan motoneuron) akibat daripada neurofibrogenesis dan ketidakfungsian mitokondria telah membantutkan proses regenerasi neuron. Taxol, sejenis alkaloid diterpene, telah didapati mempunyai kesan untuk melambatkan neurofibrogenesis melalui penstabilan mikrotubul dan mendorong regenerasi axon. Kajian ini telah dirancang untuk menentukan kesan pelindung-saraf Taxol secara infusi intrathecal dalam membendungi kematian motoneuron dan ketidakfungsian mitokondria selepas kecederaan avulsi brachial plexus.

Bahan dan Kaedah

Tikus Sprague-Dawley telah dibahagikan kepada dua kumpulan utama, Rawatan dan Kontrol (setiap kumpulan utama N=32). Kecederaan avulsi pada akar saraf brachial telah ddilaksanakan ke atas setiap tikus. Kumpulan Rawatan telah menerima infusi Taxol secara intrathecal selama 5 hari (256ng/hari) melalui pump infusi mikro,

sedangkan kumpulan Kontrol telah menerima saline biasa. Saraf tunjang cervikal telah dikeluarkan pada 1, 2, 4 dan 6 minggu selepas kecederaan (setiap kumpulan kecil n=8). Bilangan motoneuron hidup dan motoneuron nNOS-positif pada ventral horn yang cedera telah ditentukan dengan histokimia NADPH-d dan penwarnaan neutral red. Fungsi mitokondria pada ventral horn yang cedera telah ditentukan dengan histokimia CcO dan densitometer. Independent *t*-test telah digunakan untuk mengesan perbezaan di antara kumpulan-kumpulan kajian pada jangkamasa tertentu selepas kecederaan.

Keputusan

Berbanding dengan kumpulan Kontrol, kumpulan Rawatan telah menunjukkan penurunan signifikan dalam ekspresi nNOS 2 pada minggu 2, 4, dan 6 selepas kecederaan, serta kenaikan signifikan fungsi mitokondria pada minggu 4 dan 6 selepas kecederaan. Kadar kehidupan motoneuron juga meningkat secara signifikan pada minggu 2, 4, dan 6 selepas kecederaan untuk tikus-tikus yang menerima rawatan Taxol.

Kesimpulan

Taxol didapati mempunyai kesan pelindung-saraf untuk membendung degenerasi motoneuron saraf tunjang berikutan kecederaan avulsi brachial plexus secara perencatan ekspresi nNOS dan mengelakkan ketidakfungsian mitokondria.

CHAPTER 1: INTRODUCTION

Brachial plexus injuries in adults are commonly caused by motor-vehicle accidents or self-accidents such as fall from height. The surgical management of brachial plexus injury consists of nerve repair and nerve grafting for extraforaminal nerve root or trunk injury, and neurotization or nerve transfer for nerve root avulsion injury (Songcharoen, 2008). However, the outcome of brachial plexus reconstruction and the restoration of shoulder and elbow function are often poor in spite of the sophistication of the various methods used (Blaauw *et al.*, 2008; Songcharoen, 2008).

The degeneration and death of a major proportion of the innervating neuronal pool is likely to be the most fundamental neurobiological barrier to functional restitution because survival is an essential prerequisite for regeneration (De Palma *et al.*, 2008). Root avulsion of the brachial plexus causes an oxidative stress reaction in the spinal cord and induces gradual spinal motoneuron death. Loss of neurotrophic factors support secondary to axonal transport failure also leads to spinal motoneuron death as well (Yin *et al.,* 2008). After brachial root avulsion in rats, about 20% of the spinal motoneurons died at 2 weeks after the injury and about 50% of them were lost at 4 weeks after the injury (Wang *et al*., 2010).

Animal studies showed de novo expression of neuronal nitric oxide synthase (nNOS) in injured spinal motoneurons. The time course and density of nNOS expression were correlated with the severity of spinal motoneuron death following brachial root avulsion injury, in which the oxidant peroxynitrite ($ONOO^-$) played an important

role. Maximum expression of nNOS in the injured spinal motoneurons was observed between 2-3 weeks following avulsion injury (Yang *et al.*, 2008).

After spinal root avulsion injury, the neurotrophic factors (brain-derived neurotrophic factor, BDNF, and glial cell-derived neurotrophic factor, GDNF) are released from the innervated target site, taken up by the nerve terminal, and transported to the cell body via retrograde axonal transport. These factors are important for axonal regeneration and survival of the injured spinal motoneurons (Sendtner and Beck, 2009). However, scarring and fibrosis of the injured nervous tissue may impair axonal regeneration and eventually affect the neurotrophic factors transportation along the axon (Hellal *et al.*, 2011) and lead to the motoneurons death subsequently.

In addition, exposure to nitric oxide (NO) and reactive oxygen species (ROS) following post-traumatic inflammatory process would lead to neuronal mitochondrial dysfunction, especially the complex IV (cytochrome c oxidase) activity which serves as the main source for neuronal energy production (Mahad *et al.*, 2009). Thus, deprivation of both motoneurons energy demands and interference of the neurotrophic factors retrograde axonal transport to the cell bodies have reduced the survival rate of spinal motoneurons following the brachial plexus avulsion injury.

CHAPTER 2: LITERATURE REVIEW

2.1 The Anatomy of Brachial Plexus in Rats

Most of the experimental studies on spinal cord and peripheral nerve injuries were using rats sample. Although there was a clear homology with the elements of the brachial plexus in the rat and in man, the origin of the different terminal and collateral branches were found to be different in these two species (Pais et al., 2010). The rat the spinal cord is made up of 34 segments: 8 cervical (named C1 to C8), 13 thoracic (T1 to T13), 6 lumbar (L1 to L6), 4 sacral (S1 to S4), and 3 coccygeal (Co1 to Co3). A brachial plexus morphology study in 30 rats by Angelica-Almeida *et al*. (2013) demonstrated that brachial plexus was composed of branches originating from the ventral aspect of C4 to C8 and T1. In 57% of cases, the ventral aspect of T2 established an anastomosis with the ventral aspect of T1, thus contributing to the formation of the brachial plexus. This branch from T2, as well as the branch from C4 to the brachial plexus, was smaller than the remaining branches that formed the roots of the plexus. The brachial plexus roots emerged between the anterior and middle scalene muscles, forming a flattened plexus below the clavicle. The lateral, medial and posterior cords of the plexus were not clearly seen compared to those in human. The median nerve was the thickest terminal branch of the brachial plexus in rats, and almost always originated from three different roots. A branch from the second and/or the third intercostal nerve to the medial brachial and medial antebrachial cutaneous nerves was found in 87% of cases. Figure 2.1 shows the

schematic diagram of the anatomy of brachial plexus in rat. Plate 2.1 shows the branches of brachial plexus in rat and their association with the major arterial trunks.

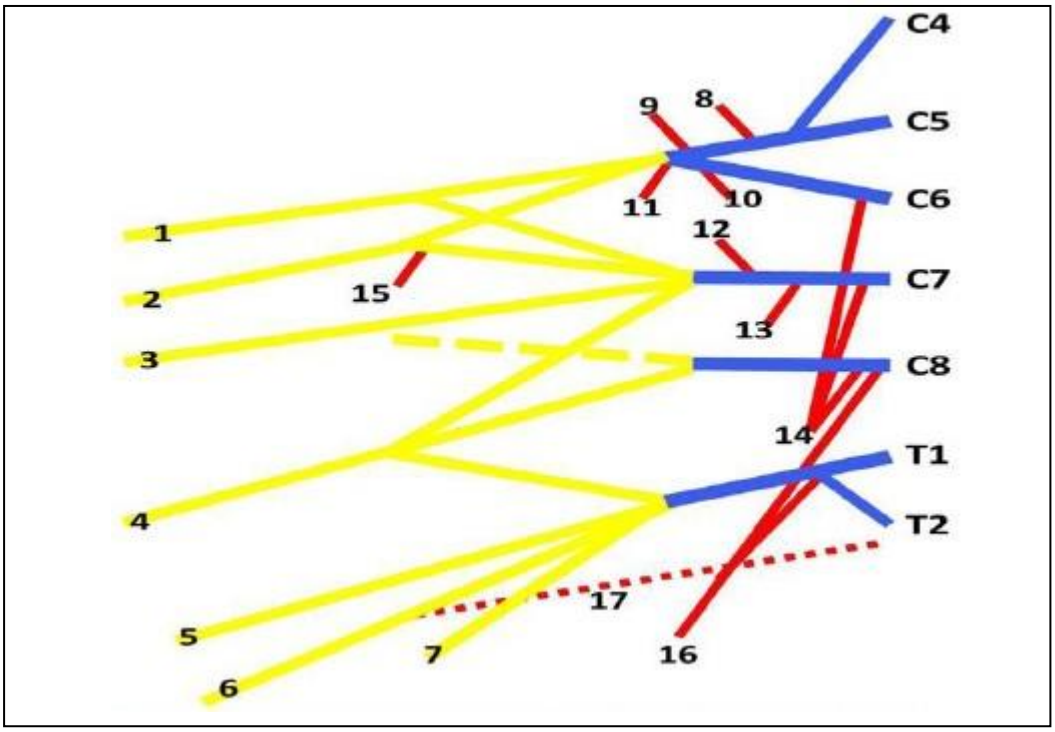

Figure 2.1: Schematic diagram of the brachial plexus in rat

(Source: Angelica-Almeida *et al*.,2013)

1- Axillary nerve; 2- Musculocutaneous nerve; 3- Radial nerve; 4- Median nerve; 5- Ulnar nerve; 6- Medial brachial cutaneous nerve; 7- Medial antebrachial cutaneous nerve; 8- Dorsal scapular nerve; 9- Suprascapular nerve; 10- Nerve to subclavius muscle; 11- Upper subscapular nerve; 12- Lower subscapular nerve; 13- Thoracodorsal nerve; 14- Long thoracic nerve; 15- Lateral pectoral nerve; 16- Medial pectoral nerve.

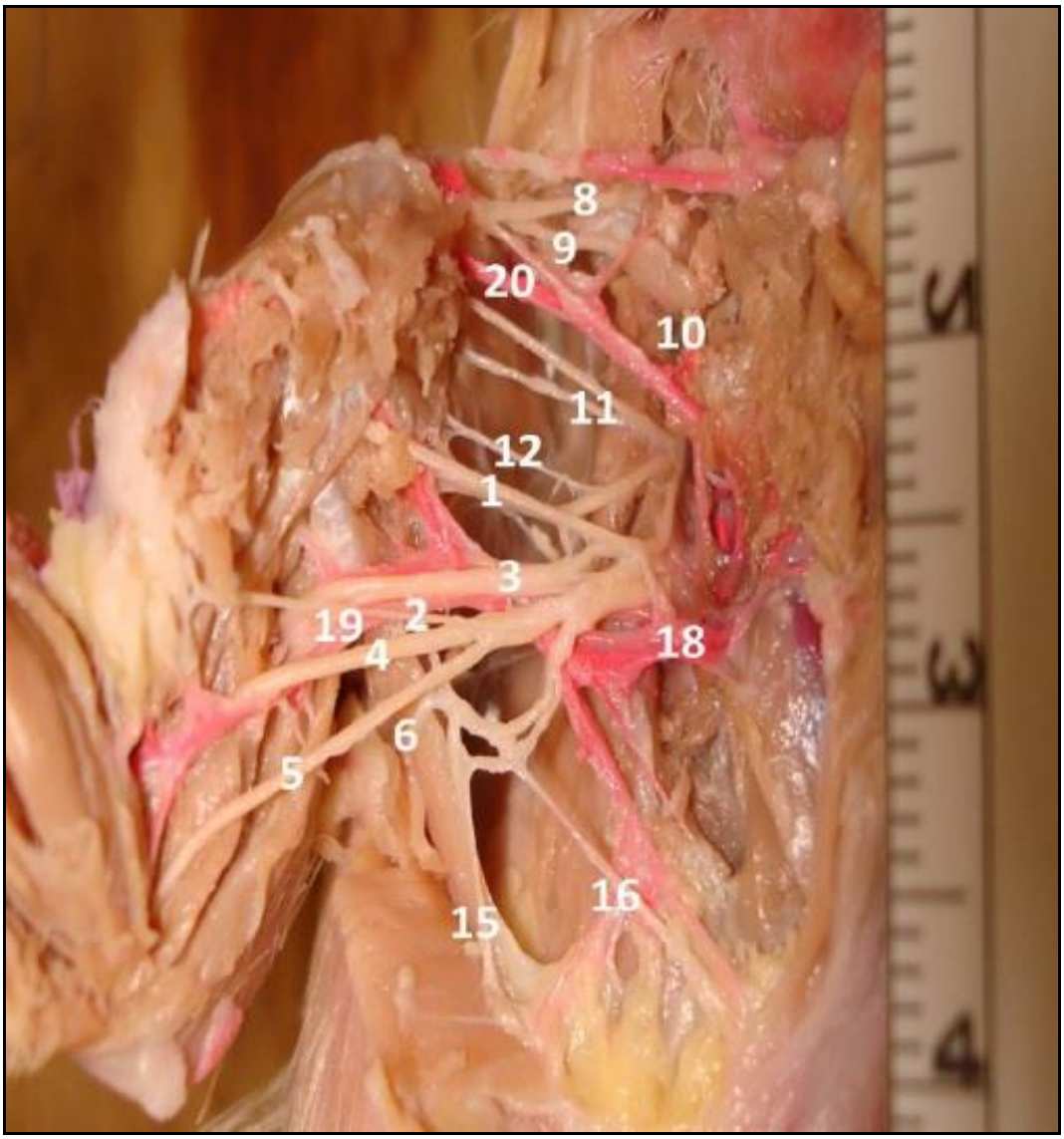

Plate 2.1: Brachial plexus and the arterial supply in rat

(Source: Angelica-Almeida *et al*.,2013)

Ventral aspect of a right forepaw dissection showing several of the terminal and collateral branches of the brachial plexus, and their association with several major arterial trunks (4X magnification). 1- Axillary nerve; 2- Musculocutaneous nerve; 3- Radial nerve; 4- Median nerve; 5- Ulnar nerve; 6- Medial brachial cutaneous nerve; 8- Dorsal scapular nerve; 9- Suprascapular nerve; 10- Nerve to subclavius muscle; 11- Upper subscapular nerve; 12- Lower subscapular nerve; 15- Lateral pectoral nerve; 16- Medial pectoral nerve; 18- Axillary artery; 19- Brachial artery; 20- Acromial arterial trunk.

The arterial supply to the BP plexus was seen to derive directly or indirectly from the vertebral, axillary, brachial, and median arteries, as well as from arteries arising directly from the aortic arch, and from the acromial and cervical arterial trunks. The venous drainage followed a similar path to the homonymous arterial structures, draining ultimately in the median, brachial, axillary and cephalic veins

2.2 The Anatomy of Ventral Roots, Dorsal Roots and Spinal Cord-Spinal Nerve Junction in Rats

The spinal cord is divided into spinal cord segments. Each segment gives rise to paired spinal nerves. Ventral and dorsal spinal roots arise as a series of rootlets (Plate 2.2). A spinal ganglion is present distally on each dorsal root. Each ventral root (also named the anterior root, radix anterior, radix ventralis, or radix motoria) is attached to the spinal cord by a series of rootles that emerge from the ventrolateral sulcus of the spinal cord. Unlike the dorsal root fibers that are arranged in a neat line at their emergence from the spinal cord, ventral root fibers form an elliptical area named the anterior root exit zone (AREZ). The ventral roots predominantly consist of efferent somatic motor fibers (thick alpha motor axons and medium-sized gamma motor axons derived from nerve cells of the ventral column (Watson *et al.*, 2009). Each dorsal root (also known as the posterior root, radix posterior, radis dorsalis or radiz sensoria) is attached to the dorsolateral sulcus of the spinal cord by a series of rootlets arranged in a line, the dorsal root entry zone (DREZ). In the experimental study using rat model, the avulsion surgery was done by separating both the ventral and dorsal roots at the junction between their attachment to the spinal cord, which were the AREZ and DREZ (Watson *et al.*, 2009).

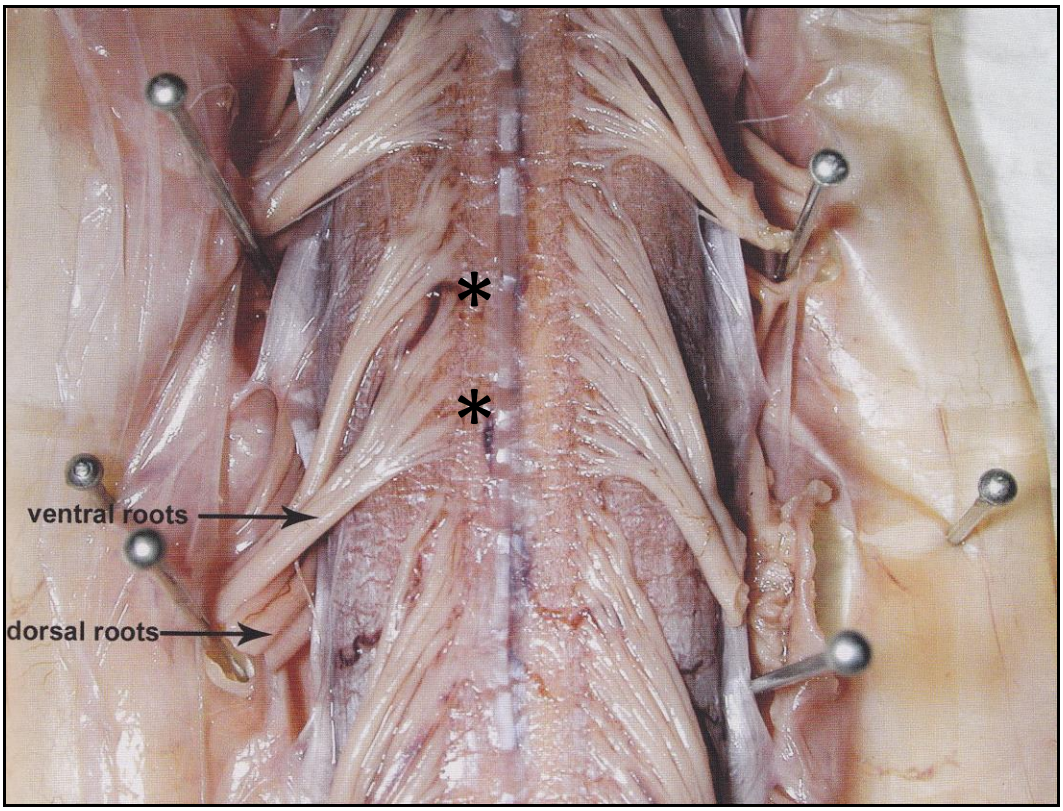

Plate 2.2: Dorsal and ventral roots of spinal nerves

(Source: Watson *et al.*, 2009)

This is a dissection showing the ventral surface of the spinal cord and the ventral and dorsal rootlets. Groups of rootlets form the dorsal and ventral roots of each spinal nerve. The dura and arachnoid have been removed to expose the spinal cord. The junction between spinal cord and ventral root (anterior root exit zone, AREZ) is labeled **.

The spinal cord gray matter is made up of neuronal cell bodies, dendrites, axons, and glial cells. The neurons are mostly multipolar, but vary greatly in size. Microscopic analysis of the spinal gray matter reveals ten different cytoarchitecture layers of cells from dorsal to ventral, which are the laminae of Rexed. Lamina IX, located at the base of the ventral horn, is the site of the motoneurons of the spinal cord (Plate 2.3). The α-motoneurons, whose axons innervate striated muscles, are the largest of all cells in the spinal cord and are usually star-shaped. Amongst these large cells, some small γ-motoneurons which innervate contractile elements of the muscle spindles are also found (Watson *et al.*, 2009).

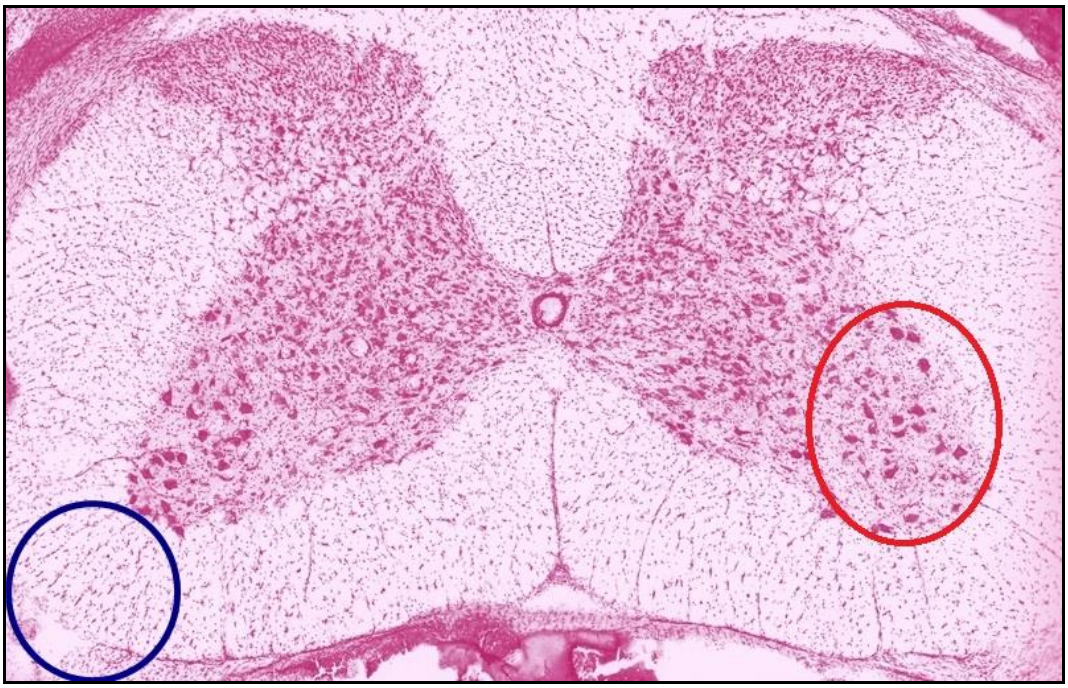

Plate 2.3: Histological section across C7 spinal segment in rat

(Source: Watson *et al.*, 2009)

The red circle indicates the location of lamina IX. The blue circle indicates the anterior root exit zone, AREZ.

2.3 Brachial Plexus Injury

Brachial plexus injury (BPI) is a severe neurologic injury that causes significant functional impairment of the affected upper limb. The most common cause of BPI is road traffic accidents with most of the victims being young males (Shin *et al.*, 2010; Songcharoen, 2008). Other reported traumatic causes include sport injuries, accidents at work, penetrating injuries, gunshot wounds, and iatrogenic causes (for example, patient malpositioning during surgery). Another common cause of BPI is birth palsy (Shin *et al.*, 2010). The majority of obstetric BPI involves the upper brachial plexus, for example the Erb or Duchenne palsy. Lower type obstetric BPI (Klumpke palsy) is rare. Tumors, irradiation, and congenital abnormalities such as cervical ribs can be nontraumatic causes of brachial plexopathy.

BPI is caused by severe traction force exerted on the upper limb, resulting in complete or partial motor paralysis. An upper brachial plexus lesion involves spinal nerves C5 and C6 and leads to paralysis of the shoulder muscles and biceps. When the damage extends to spinal nerve C7, some of the wrist muscles are also impaired. A lower brachial plexus lesion involves spinal nerves C8 and T1 leads to paralysis of the forearm flexor and the intrinsic muscles of the hand (Cardenas-Mejia *et al.*, 2008).

2.3.1 Classification of Brachial Plexus Injury

Adult BPI remains a dilemma to many surgeons, especially when planning to reconstruct cases of total root avulsion. Different degrees and different levels of injury require different approaches of reconstruction. Chuang (2010) classified brachial plexus injury into 4 levels as shown in Table 2.3.1.

Table 2.3.1: Chuang's classification of brachial plexus injury

Type of Injury	Description
Level 1	Preganglionic root injury including spinal cord, rootlets, and root injuries.
Level 2	Postganglionic spinal nerve injury limiting the lesion to the interscalene space and proximal to the suprascapular nerve.
Level 3	Preclavicular and retroclavicular BPI including trunks and divisions.
Level 4	Infraclavicular BPI including cords and terminal branches proximal to the axillary fossa.

2.3.2 Management of Brachial Plexus Injury

Factors that would determine the choices of treatment in BPI include: i) the degree of damage, ii) the site of injury, iii) the number of roots involved, iv) the time interval between the injury and the surgical procedure, and V) the patient's age and occupation. Among these, the degree of damage and the site of injury are the most important factors (Doi, 2008). Management of BPI can be either conservative or surgical. Representative surgical procedures include neurolysis, nerve grafting, nerve transfer, and other reconstructive procedures involving the transplantation of various structures (Cardenas-Mejia *et al.*, 2008; Doi, 2008).

Preganglionic injuries are usually considered not amenable to repair; consequently, the functions of some denervated muscles are restored with nerve transfers. In nerve transfer, the donor nerve is attached to the ruptured distal stump, sacrificing the original function of the nerve for more beneficial functions in the upper limb (Rankine, 2010). It is generally agreed that the top priority of nerve repair is restoration of biceps muscle function and the second goal is reanimation of shoulder function (Chuang, 2010; Doi 2008). Intercostal nerve is frequently used as the donor nerve transferred to the musculocutaneous nerve to regain elbow flexion. Functional recovery of the shoulder is largely achieved with transfer of spinal accessory nerve to the suprascapular nerve. Feng *et al.* (2010) reported nerve transfer of contralateral C7 to lower trunk via a subcutaneous tunnel across the anterior surface of chest and neck in 4 patients with total brachial plexus avulsion and the procedure was proved to be a safe and feasible. Compared with the traditional transfer of the contralateral

C7 to the median nerve, it might help patients gain better restoration of wrist flexion, finger flexion, and hand sensation.

In postganglionic injury with disruption of the nerve fibre, it is repaired with nerve grafting. The damaged segment is excised and nerve autograft is placed between the two nerve ends (Chuang, 2010). If the postganglionic lesion in continuity is non-degenerative or the fascicles are still intact, spontaneous recovery is usually expected with conservative management. Whereas postganglionic lesion in continuity of degenerative type with damaged fascicles, it is treated with nerve grafting. Patient with severe BPI should undergo an appropriate reconstructive procedure before denervated muscles become irreversibly atrophy, otherwise the patient will no longer a good candidate for primary nerve repair (Rovak and Tung, 2009).

2.3.3 Surgical Outcome in Brachial Plexus Avulsion Injury

Avulsion of brachial roots from the spinal cord is a devastating injury with a bleak prognosis. Patients with preganglionic type of BPI have been reported with poorer surgical outcome (Rovak and Tung, 2009; Terzis *et al.*, 2009). Experimental studies with implantation of avulsed ventral roots in rats, cat, and chimpanzee were shown to promote motor recovery. This is because axons from spinal cord motoneurons can grow into ventral roots and peripheral nerves (Yang et al., 2008). However, the clinical usefulness of reimplantation in patient with brachial roots avulsion is not clear. The current practice of surgical repair of brachial plexus avulsion by reimplantation of avulsed roots via a peripheral nerve graft provides a small degree of motor recovery; however, useful hand function is mostly not restored in adult

patients. There is no recovery of sensation, although pain is usually alleviated for the reimplanted segments (Rankine, 2010).

2.4 Molecular Pathogenesis of Post Traumatic Spinal Motoneurons Degeneration

Much of the spinal tissue degeneration that occurs following spinal cord injury (SCI) is due to secondary injury processes that are triggered by the primary mechanical trauma. The events of the secondary injury phase can be divided into early and delayed stages (Kuzhandaivvel et al., 2011; Donnelly and Popovich, 2008). The early stage of secondary injury is thought to start with excitotoxic damage due to massive release of glutamate together with a pathological cascade comprising nitric oxide, free oxygen radicals, and metabolic dysfunction due to ischemia/hypoxia, energy store collapse, acidosis, and edema triggered by loss of vascular tone autoregulation. Later, macrophage infiltration and initiation of glial scar occur (Hagg and Oudega, 2008; Donnelly and Popovich, 2008).

This early stage of secondary injury starts minutes after primary insult and can lasts up to weeks after injury. Extracellular glutamate levels are known to increase transiently within the first 3 hours after SCI, with a likely second wave of glutamate release 2 to 3 days after injury, probably due to delayed myelin destruction that compromises nearby axon integrity. The over-stimulation of glutamate receptors has been reported to contribute to neuronal and glial cell death after experimental SCI (Hagg and Oudega, 2008).

The delayed stage of secondary injury starts 2 weeks to 6 months after the insult, glial scarring continues together with intraspinal cyst formation. Even later, profound pathological changes affect spinal networks through Wallerian degeneration, demyelination, and aberrant plasticity with circuit rewiring leading to dysfunction like chronic pain and spasticity (Kuzhandaivel et al., 2011; Donnelly and Popovich, 2008).

2.4.1 Nitric Oxide Synthase

Nitric oxide (NO) is a gaseous neurotransmitter in central nervous system (CNS) and peripheral nervous system (PNS), and is able to diffuse across the cell membrane. NO is involved in several physiological processes including smooth muscle relaxation, inflammation, vasodilatation, neurogenesis, synaptic plasticity, long-term potentiation, and nociceptive transmission (Freire *et al.*, 2009). Low levels of NO production are important in prevention of cells apoptosis. However, elevated levels secondary to increased NO production result in direct cytotoxicity (De Palma *et al.*, 2008). Reaction between this NO and superoxide radicals (O_2^-) will produce a type of reactive oxidative species (ROS) called peroxynitrite (PN). PN has been proposed to be a key contributor to post-traumatic oxidative damage, mainly because of its highly reactive decomposition products nitrogen dioxide ($^{\cdot}NO_2$), hydroxyl radical ($^{\cdot}OH$) and carbonate radical ($CO_3^{\cdot -}$). These PN-derived radicals can oxidize proteins and nitrate tyrosine residues, induce cell membrane lipid peroxidation, cause single-strand DNA breaks, and also inhibit mitochondrial respiration (Alvarez and Radi, 2009).

The enzyme nitric oxide synthase (NOS) catalyzes the production of nitric oxide from L-arginine and oxygen, and different isoforms of the enzyme exist in different tissues. NO is constitutively produced by neuronal NOS (nNOS) and endothelial NOS (eNOS) in a calcium-dependent manner, and is also formed by an inducible form of the enzyme (iNOS), which may have detrimental effects on the cell (Miclescu and Gordh, 2009). Neuronal NOS is found in both the CNS and PNS. Neuronal NOS has been specifically localized to spinal cord dorsal horn neurons and dorsal root ganglia cells, and is upregulated in conditions of inflammatory and neurogenic pain (Schmidtko *et al.*, 2009).

Peripheral nerve lesions and spinal cord injury have been shown to induce upregulation of all NOS isoforms, as demonstrated by NADPH-diaphorase histochemistry. Such increases in NOS expression result in enhanced expression of NO in the nerve microenvironment and induce mitochondrial dysfunction as well as neuronal cells death (Yang *et al.*, 2008).

2.4.2 Free Radical-Induced Lipid Peroxidation

Extensive evidence has shown that free radical-induced lipid peroxidation (LP) plays a major role in the acute pathophysiology of SCI (Alvarez and Radi, 2009). LP begins with the oxidation of polyunsaturated fatty acids (e.g., arachidonic, linoleic, and docosahexaenoic acids) in the cell, or in membrane phospholipids at their allylic carbon. The peroxidized polyunsaturated fatty acids undergo phospholipase-mediated hydrolysis and consequent disruption of the membrane phospholipid architecture, and loss of the function of phospholipid-dependent enzymes, ion

channels, and structural proteins. However, in addition to LP-induced membrane damage, the peroxidized fatty acids ultimately give rise to aldehydic breakdown products, including 4-hydroxy-2-nonenal (4-HNE) and 2- propenal (acrolein). These aldehydes are highly reactive with cellular proteins via Schiff base and Michael addiction reactions with basic (for example, lysine and histidine) and sulfhydryl (for example, cysteine) containing amino acids (Stevens and Maier, 2008). These reactions have been shown to impair the function of a variety of cellular proteins, which could also contribute to post-traumatic secondary injury and the associated pathophysiology. Sources of post-traumatic reactive oxygen species (ROS) that result in toxic LP-inducing secondary injury include iron-dependent Fenton reactions, which result in hydroxyl radical (·OH) production and peroxynitrite (PON)-derived free radicals (·OH, $\cdot NO_2$, and $\cdot CO_3$)

2.5 Mitochondrial Oxidative Phosphorylation Activity

The predominant physiological function of mitochondria is the generation of ATP by oxidative phosphorylation. Additional functions include the generation and detoxification of reactive oxygen species, involvement in some forms of apoptosis, regulation of cytoplasmic and mitochondrial matrix calcium, synthesis and catabolism of metabolites and the transport of the organelles themselves to correct locations within the cell (Ramzan *et al.*, 2010).

Almost all functions of mitochondria are either directly or indirectly linked to the working of oxidative phosphorylation machinery and energy coupling. Most part of this machinery is in the inner mitochondrial membrane and comprises the four

electron transfer chain complexes (complexes I, II, III, and IV), ATP synthase (complex V), NADH dehydrogenase (ubiquinone), and cytochrome c as electron carriers (Magrane and Manfredi, 2009; Brand and Nicholls, 2011). Complex IV or cytochrome c oxidase (CcO) is the terminal enzyme of the electron transport chain, which catalyzes the final step of electron transfer from reduced cytochrome c to oxygen to produce water (H_2O). CcO is also one of the three proton pumps along with complexes I and III that generate the proton gradient across the inner mitochondrial membrane, which powers the ATP synthesis. A very common approach to address mitochondrial bioenergetics dysfunction is to measure the expression, concentration or maximum activity of a few candidate electron transport complexes or metabolic enzymes, such as complex I and complex IV (Acin-Perez *et al.*, 2011; Brand and Nicholls, 2011).

2.5.1 Cytochrome c Oxidase

CcO in mammals contains 13 subunits of which the 3 catalytic subunits are encoded by the mitochondrial genes. The remaining 10 subunits, which are synthesized in cytosol and imported into mitochondria, are coded by the nuclear genome. These subunits are believed to provide structural stability to the complex as well as involved in the regulation of enzyme activity. CcO contains two heme groups (heme a and a3) and two copper centers (Cu^{2+} A and Cu^{2+} B) as catalytic centers and handles more than 90% of molecular O2 respired by the mammalian cells and tissues. CcO acts as the rate-limiting step of the respiratory chain and its activity is an indicator of the oxidative capacity of the cells (Acin-Perez *et al.*, 2011).

2.5.2 Mitochondrial Transportation

Mitochondrial function, including aerobic production of ATP and calcium buffering, is vital to the health of the neuron, and therefore neurons must have a proper intracellular distribution of mitochondria. Mitochondria are enriched at sites of high ATP utilization and Ca^{2+}-buffering demands, such as cell bodies, nodes of Ranvier, and synaptic terminals (Reeve et al., 2008). Mitochondria are actively transported to areas of high metabolic demand by the motors kinesin and dynein in a calcium regulated process involving the protein Milton and the mitochondrial Rho GTPase. In addition, mitochondria are also transported along the cell processes in variable speed with intracellular signalling (Magrane and Manfredi, 2009). The direction of mitochondrial transport has been proposed to correlate with their bioenergetics state: mitochondria with normal membrane potential tend to move toward the periphery (anterograde movement), whereas loss of membrane potential results in increased retrograde transport (Srinivasan and Avadhani, 2012). Defects in mitochondrial transport would lead to altered distribution of mitochondria along the axon, in turn leading to an inability to meet local ATP demands and/or toxic changes in calcium buffering.

2.5.3 Mitochondrial Dysfunction

The mitochondria are present in a physiological environment, are exposed to a relevant mix of substrates and ions and interact with the cytoplasm, plasma membrane and other organelles and cell structures. The structure and function of the enzyme are affected in a wide variety of diseases including cancer, neurodegenerative diseases, myocardial ischemia or reperfusion, bone and skeletal diseases, and diabetes (Cooper and Brown, 2008). Except in cases of genetic defects, it is commonly seen that mitochondrial dysfunction is a cumulative effect of failure of more than one complex of the electron transport chain (Mahad *et al.*, 2009). Some of the common mechanisms of CcO dysfunction include assembly defects, covalent modifications and loss of subunits, disassembly of super complex organization and direct inhibition of enzyme activity. The impact of these events includes energy crisis due to lower ATP production, lactic acidosis, and increased formation of ROS in mitochondria (Ramzan *et al.*, 2010).

Four different gases, nitric oxide (NO), carbon monoxide (CO), hydrogen sulfide (H_2S), and hydrogen cyanide bind to CcO and invariably inhibit the enzyme activity. NO has been established as an important second messenger, which is involved in diverse physiological and pathological functions. Although soluble guanyl atecyclase is one of its most prominent targets, NO interacts with metal centers of many proteins. The inhibition of CcO by NO is thought to be reversible. Since O_2 and NO compete for the same binding site in CcO, endogenously generated NO can reach concentrations that are inhibitory to CcO under physiological oxygen levels (Srinivasan and Avadhani, 2012).

2.5.4 Mitochondria Dysfunction and Oxidative Stress

Mitochondria are the principal source of cellular reactive oxygen species (ROS). ROS, particularly superoxide anions, are formed invariably as by- products of the electron transport chain and other redox reactions in mitochondria through one-electron reduction of molecular oxygen (O_2). Superoxide is then converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs), present both within the mitochondria and in the cytosol (Ferreira *et al.*, 2010). Depending on their type and rate of production, ROS have both physiological roles and pathological effects in the context of mitochondrial as well as whole cell function. Excessive production of ROS and the associated cytotoxic effects are generally called oxidative stress (Kawamata and Manfredi, 2010; Waldbaum and Patel, 2010). Peroxidation of membrane lipids, direct oxidation of amino acids, and oxidative cleavage of peptide bonds in proteins and DNA damage are some of the hallmarks of oxidative stress and are responsible for many of the disease symptoms. Although several redox reactions take place in mitochondria, only a few of them have been shown to generate detectable oxygen free radicals. While complexes I and III are the major sites of ROS formation, recent reports show that complex II can readily generate superoxide radicals in the absence of electron acceptors. A volume of evidence suggests that CcO dysfunction is invariably associated with increased mitochondrial ROS production and cellular toxicity (Zambonin *et al.*, 2010).

2.6 Axonal Transportation and Regeneration

The unique morphology of neurons, highly polarized cells with extended axons and dendrites, makes them particularly dependent on active intracellular transport. The transport of proteins, RNA, and organelles over long distances requires molecular motors that operate along the cellular cytoskeleton (Perlson *et al.*, 2010; Stevens and Maier, 2008). Two major roles for axonal transport are supply/clearance and long-distance signalling. Supply of newly synthesized proteins and lipids to the distal synapse maintains axonal activity, whereas misfolded and aggregated proteins are cleared from the axon by transport to the cell soma for efficient degradation. Active transport of mitochondria also supplies local energy needs. The second major role for active transport is the communication of intracellular signals from the distal axon to the soma, allowing the neuron to respond to changes in environment. While defects in either supply or clearance can readily be predicted to be deleterious to the health of the neuron, there has been a growing appreciation that the propagation of stress-signaling along the axon could be a key neurodegenerative pathway leading to cell death (Yin *et al.*, 2008).

The proximal cause of cell death in affected neurons possibly is that inhibition of transport leads to defects in the localization or delivery of essential cargos. For example, failure to deliver mitochondria to areas of need could induce cell death through energy deprivation. Or, disruption of lysosomal and/or autophagosome motility could lead to the toxic build-up of aggregated proteins or defective organelles (Magrane and Manfredi, 2009). Another hypothesis is that the key defect

in axonal transport is not a disruption in bulk supply/clearance, but instead is an alteration in cell signalling (Amiri and Hollenbeck, 2008).

Axons in the central nervous system (CNS) do not regrow after injury, whereas lesioned axons in the peripheral nervous system (PNS) regenerate. After injury, the formation of a growth cone at the tip of a transected axon is a crucial step during subsequent axonal regeneration. On the contrary, lesioned CNS axons form swellings termed "retraction bulbs" at the tip of their proximal stumps, which are oval structures and lack a regenerative response (De Vos *et al.*, 2008). Growth cones contain the machinery for movement and axonal extension consisting of a complex interplay of different intracellular events. For example, mitochondria concentrate in the tip of the growing axon to provide energy necessary for axon formation. Axon growth also depends on continuous membrane supply from the soma to support the surface expansion of the growing axon. Notably, microtubules and their dynamic rearrangements are essential for axon outgrowth. Retraction bulbs of injured CNS axons increase in size over time, whereas growth cones of injured PNS axons remain constant. Retraction bulbs contain a disorganized microtubule network, whereas growth cones possess the typical bundling of microtubules. Microtubules play a key role in axonal growth and guidance. They form the backbone of the axonal shafts and core domain of growth cones, giving stability to those structures and enabling organelle transport (Mahad *et al.*, 2009). In addition, the dynamic microtubules protrude through the peripheral regions of growth cones, enabling axon elongation. Disruption of microtubules in growth cones transforms them into retraction bulb-like structures whose growth is inhibited. Thus, the stability and organization of

microtubules define the fate of lesioned axonal stumps to become either advancing growth cones or non-growing retraction bulbs.

2.7 Neurotrophic Factors

Study by Yin *et al.* (2008) showed that motor nerves are superior to sensory nerves for promoting motoneuron survival and axonal regeneration after root avulsion. This is partly caused by the higher expression of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in motor nerves.

Motoneurons require neurotrophic factors for survival during embryonic development and after injury in adult animals (Yin *et al.*, 2008). Neurotrophic factors for motoneurons are classified into families according to their structures. They include neurotrophins, cytokines, the transforming growth factor beta superfamily (TGF-β), and many others (Sendtner et al., 2009).

BDNF and GDNF are well-known neurotrophic factors for motoneurons in the neurotrophin and transforming growth factor-β families, respectively. The BDNF signals through tropomyosin receptor kinase B (TrkB) receptor, and/or low affinity nerve growth factor receptor (p75 neurotrophin receptor), whereas GDNF uses GDNF family receptors (GFR-α1 and c-ret). They promote motoneuron survival both in vitro and in vivo. Numerous studies have demonstrated that they also promote axonal regeneration (Yin *et al.*, 2008).

2.8 Taxol

Taxol (also known as Paclitaxel) has been approved by the U.S. Food and Drug Administration as an chemotherapeutic agent for the treatment of ovarian and breast cancer (Piccart *et al.*, 2010; Rowinsky *et al.*, 2009). Currently, it is also being used to treat other tumors including non-small cell lung carcinoma and Kaposi's sarcoma. It is originally derived from the bark of the western yew tree called *Taxus brevifolia*. Compared to other oncogenic agents, the clinical development of taxol progressed slowly because of the small amounts of drug obtainable from the crude bark extract and its poor water solubility (Piccart *et al.*, 2010).

Taxol is a diterpene alkaloid and it's chemical name is 5β,20-Epoxy-l,2α,4,7β,10β,13α-hexahydroxytax-l l-en-9-one 4,10-diacetate 2-benzoate 13-ester with (2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine. It is a white to off-white crystalline powder with the empirical formula $C_{47}H_{51}NO_{14}$ and a molecular weight of 853.9. It is highly lipophilic and melts at around 216-217° C (Sparreboom *et al.*, 2008). Molecular structure of Taxol is shown in Figure 2.8.