

THE NEUROPROTECTIVE ACTIONS OF QUERCETIN

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In the Department of Anatomy and Cell Biology
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By

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ABSTRACT

Trauma-induced spinal cord injury (SCI) is the most prevalent form of spinal cord injury affecting over 80% of the 36,000 Canadians living with this condition. The pathophysiological profile of traumatic SCI consists of an initial stage of direct damage followed by a series of secondary events, including reduced blood flow and increased generation of free radicals that leads to excitotoxicity, oxidative stress, hemorrhagic necrosis, inflammation, and apoptosis. We examined the hypotheses that delayed administration of the flavonoid quercetin inhibits the propagation of secondary events and promotes functional recovery after traumatic SCI by inhibiting inflammatory processes and signaling pathways that promote apoptosis and thereby promoting axon survival. To determine whether delayed quercetin treatment promoted functional recovery following SCI, male Wistar rats were subjected to a spinal cord compression injury by application of a 50 g modified aneurysm clip at the mid thoracic cord level. A treatment regimen of 75 μmol quercetin per kg rat or saline only (controls) was administered for a period of 3 days, 1 week or 2 weeks beginning at 2 weeks post surgery. Delayed quercetin treatment improved locomotion in injured animals although with severe deficit. To determine whether improved functional outcome correlated with improved tissue preservation and reduced scarring, we performed histological examinations at the injury site. In saline treated animals, at 8 weeks post injury we found over 80% of tissue loss with the majority of the remaining cells undergoing apoptosis. However, with 2 weeks delayed quercetin treatment, at least 50% of the tissue was still present at 8 weeks post surgery with a significant reduction of apoptosis. Quercetin treated animals also showed a reduction of reactive gliosis. To determine which intracellular signaling pathways may mediate the protective effects of quercetin, we carried out Western blots and immunocytochemical analyses of a number of potential pro-apoptotic pathways. We found that quercetin reduced the levels of the phosphorylated (activated) forms of the MAPK p38, ERK 1/2 (p42/44) and SAPK/JNK seen after SCI. We conclude that delayed quercetin treatment likely rescues neurons that would otherwise have died between the third and sixth weeks following injury by inhibiting apoptosis of glia cells. Quercetin may be acting via selective inhibition of kinase pathways that have been shown to be involved in apoptosis and cell growth. These findings not only reveal the protective effects of quercetin in reducing secondary damage after chronic SCI but also shed some light on some of the mechanisms underlying its actions.

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DEDICATION

To the memory of my beloved mother, Commissaire Aza'a Ngum Esther Tabien who was the strongest and most determined woman I ever met, who made enormous sacrifices so that I could get the best education and be where I am today, who always believed in me and who made me become the woman I am today. She was my confidant, my best friend and will always be my inspiration. I am thankful for having been blessed with this wonderful mother.

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LIST OF ABBREVIATIONS

ALS	amyotrophic lateral sclerosis
ASIA	American Spinal Injury Association
ATP	adenosine triphosphate
AP-1	activator protein 1
ASK1	apoptosis signaling kinase-1
BBB score	Basso Beattie Bresnahan score (for recovery of motor function)
β -APP	beta amyloid precursor protein
Caspase	cysteine dependent aspartate specific protease
CARD	caspase recruiting domain
CDR	Christopher and Dana Reeve Foundation
cIAP	cellular inhibitor of apoptosis proteins
CNS	central nervous system
cNOS	constitutive nitric oxide synthase
CPA	Canadian Paraplegic Association
COX2	cyclooxygenase-2
DAXX	death associated protein 6
DED	death effector domain
ERK 1/2	extracellular signal regulated kinase 1/2
FADD	fas-associated protein with death domain
Fe ²⁺	ferrous iron ion
Fe ³⁺	ferric iron ion
GFAP	glial fibrillary acidic protein

H ₂ O ₂	hydrogen peroxide
IAPs	inhibitor-of-apoptosis proteins
ICAM-1	intercellular adhesion molecule-1
IKK	I kappa kinase
IL-1	interleukin-1
INF- γ	interferon gamma
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
i.v	intravenous
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide (Endotoxin)
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemotactic protein-1
MHC	major histocompatibility complex
MPTP	mitochondrial permeability transition pore
MS	multiple sclerosis
NF κ B	nuclear factor kappa B
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NGF	nerve growth factor
NGFR	nerve growth factor receptor
NSCISC	national spinal cord injury statistical center
p44/44	extracellular signal regulated kinase 1/2

PARP	poly ADP-ribose polymerase
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
ROS	reactive oxygen species
RIP	ribosome inactivating protein
SAPK	stress activated protein kinase
SCI	spinal cord injury
SOD	superoxide dismutase
SODD	silencer of death domain
TGF	transforming growth factor
TGFR	transforming growth factor receptor
TNF	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
TRADD	tumor necrosis factor receptor type 1-associated death domain

LIST OF PUBLICATIONS AND PRESENTATIONS
FROM WORK PRESENTED IN THIS THESIS

ABSTRACTS and POSTERS

H E Nsoh Tabien and BHI Juurlink. A novel insight into quercetin's actions following spinal cord injury. Abstract published in Journal of Neurotrauma, June 2006. Poster presented at National Society of Neurotrauma. Saint Louis, Missouri, USA. July 2006.

H E Nsoh Tabien and BHI Juurlink. Inhibiting glia cell apoptosis to enhance neuronal recovery in spinal cord injury. Abstract published in Journal of Neurotrauma, October 2005. Poster presented at the National Society of Neurotrauma. Washington DC, USA. November 2005.

E. Schültke, ***E. Hortense Nsoh Tabien***, S. Rigley, B.H.J. Juurlink. Strategies to prevent secondary damage following spinal cord injury. Abstract published and poster presented at the Christopher Reeve Foundation Spinal Cord Symposium. Boston, MA, USA. September 2005

H E Nsoh Tabien and BHI Juurlink. Conferring neuroprotection by downregulating apoptosis and reactive gliosis with quercetin. Abstract published in Journal of Neurotrauma, September 2004. Poster presented at National Society of Neurotrauma. San Diego, California, USA. October 2004.

HE Nsoh Tabien and BHI Juurlink. The Neuroprotective effects of a flavonoid in late traumatic spinal cord injury. Abstract published and poster presented at Life Style Nutrition and Health Conference. Winnipeg, Manitoba. June 2004

B.H.J. Juurlink, E. Schültke, ***H Nsoh Tabien*** and R. W. Griebel. Quercetin: therapeutic effects following Neurotrauma. Abstract presented at the Canadian Federation of Biological Sciences (CFBS) 48th Annual Meeting. Vancouver, BC. June 2004.

H E Nsoh Tabien, Suraj Abraham, Tyler Evans, Nicole J.Cox and, BHI Juurlink. Quercetin enhances cell survival after late traumatic spinal cord injury. Abstract and Poster presented at Life Sciences Conference. University of Saskatchewan, Saskatoon. May 2004

H E Nsoh Tabien, Nicole J.Cox, Suraj Abraham, William M. Kulyk, BHI Juurlink. A bioflavonoid as a potential therapy for cutaneous scarring. Poster, Life Sciences Conference. University of Saskatchewan, Saskatoon. May 2004

BHI Juurlink, PE Paterson, RW Griebel, H Kamencic, E Schültke, S Rigley, M Kelly and ***H E Nsoh Tabien***. Decreasing secondary damage following spinal cord injury by promoting endogenous acting scavenging defense systems and by inhibiting signaling pathways that lead to inflammation and apoptosis. Poster presented at the Oxidative Stress Consortium meeting. Toronto, ON. May 2004.

HE Nsoh Tabien and BHJ Juurlink. Novel role of quercetin in chronic spinal cord injury. Abstract, published in FASEB Journal /Society of Experimental Biology and Medicine (SEBM), April 2004. Poster presented Society of Experimental Biology and Medicine conference. Washington DC. April 2004

SELECTED PRESENTATIONS

- Ameliorating spinal cord injury with quercetin** November 2007
Graduate seminar presentation at the Department of Anatomy and Cell Biology,
University of Saskatchewan, Saskatoon
- Exploring quercetin's actions in chronic spinal cord injury** July 2006
Platform presentation at the National Society of Neurotrauma,
Saint Louis Missouri, USA
- Exploring quercetin from a different angle** April 2006
Graduate seminar presentation at the Department of Anatomy and Cell Biology,
University of Saskatchewan, Saskatoon
- Brain and spinal cord injury** March 2005
Presentation at the "Brain Show" during the Brain Awareness Week,
University of Saskatchewan, Saskatoon
- Role of quercetin in chronic spinal cord injury** April 2004
Platform presentation at American Association of Anatomist,
Society of Experimental Biology conference. Washington DC, USA

1.1 Epidemiology of spinal cord injury

Spinal cord injury (SCI) is a major problem in Canada and throughout the rest of the world. A recent survey of over 33,000 households initiated by the Christopher and Dana Reeves foundation (CDR Foundation, April 2009) shows that 1.275 million Americans have had spinal cord injury and over 5.6 million live with some form of paralysis. These estimates are at least five times more for people living with spinal cord injury and 40% more for people living with paralysis than the previous estimates of roughly 250,000 and 4 million respectively (Sekhon and Fehlings, 2001; Kirshblum et al., 2002).

According to the Canadian Paraplegic Association (CPA) there are approximately 36,000 Canadians with SCI with an incidence rate estimated at 42.4 per year per million for adults aged 15-64 and 51.4 per million population for those 65 years or older (Pickett et al., 2006). SCI is predominately experienced by young adults in the second and third decade of life. Statistics from around the globe shows that of the newly injured each year, the majority (80%) are male (Jongbloed et al 2007; Go et al., 1995). In addition, of the new injuries reported in Canada, approximately 53% are paraplegic and 47% are quadriplegic (Pickett et al 2006; Ackery et al., 2004; Dryden et al., 2003; Tator et al., 1993).

The leading cause of SCI, in Canada is vehicular accidents (car and motorcycle) (41.2%), followed by falls, including industrial accidents (21.8%) while other conditions including medical causes, sports injuries and diving account for the remainder (37%) (CPA, Pickett et al., 2006; Ackery et al., 2004; Tator et al., 1993; Tator, 1983). Traumatic insult is usually caused by sudden impact, which crushes the spinal cord giving rise to a change in its normal motor, sensory, or autonomic function. Injuries to the cervical spinal cords are more common than those

of thoracic and lumbar cord, but thoracic cord injuries have been associated with a higher percentage of complete spinal cord lesions (Pickett et al 2006; Tator et al., 1993; Tator, 1983). The most common mechanism of injury to the spinal cord is fracture dislocation (Tator, 1983; Sekhon and Fehlings, 2001; Pickett et al., 2006). A fracture dislocation occurs when a portion of the vertebra is fractured and moves into the spinal canal causing damage to the cord. According to the direction of the dislocation the fracture can be classified as anterior, posterior or lateral. Most SCI are attributable to anterior fracture dislocation while posterior and lateral fractures are less common.

The total health care cost of SCI in Canada is estimated at about \$750,000,000/annum (Riis et al., 2007; Dryden et al., 2005). However, far greater than the financial burden on the health care system is the burden placed upon the individual afflicted as well as family and friends (Jongbloed et al 2007; Riis, 2007; Dryden et al., 2005). SCI causes major functional, medical, and financial effects on the injured person as well as an important effect on the individual's psychosocial well-being (Dryden et al., 2005; Tator et al., 1993; Fiedler et al., 1999; Krause et al., 1997; Kirshblum, 2002). In addition, the society as a whole incurs tremendous economic losses due to the lack of contributions from these young individuals that could have been potential sources of economic power (Strauss et al., 2008; Jongbloed et al 2007; Riis et al., 2007; Budh and Osteraker 2007). It has been reported that the employment rate for young adults who suffered from acute traumatic SCI drops to approximately half the employment rate of the same age group in the general population (Vogel et al., 1998; Anderson et al., 2000; Pflaum et al., 2006).

1.2 Research and medical advances in spinal cord injury

The past two decades have seen important advances in research as well as in emergency medical treatments and specialized care of the patient with SCI. This has led to a significant decrease in medical complications and improvement in the quality of life of patients suffering from this condition. The routine practice of urinary drainage by catheterization and proper skin care that was introduced since the early 1940s (Guttmann et al., 1949) has led to significant decrease in mortality due to sepsis and urological complication which used to be the leading cause of death in SCI patients. According to the National Spinal Cord Injury Statistical Center (NSCISC), respiratory complications are currently the most common causes of death in SCI patients followed by heart disease, subsequent trauma, and septicaemia (Shavelle et al., 2006; Furlan et al., 2008). Suicide and alcohol-related deaths are also major causes of death in patients with SCI (Turner et al., 2003; Frisbie, 1984).

An interesting trend seen in SCI is the decreasing number of complete cord lesions in both paraplegic and quadriplegic (tetraplegia) patients arriving in hospitals (Sekhon and Fehlings, 2001; Tator et al., 1993). This is in large part due to advances in emergency medical treatments of SCI which aim at prompt interventions to stop the spread of the initial lesion. Other contributing factors to this trend are an increased public awareness with widespread first aid knowledge and implementations of mandatory safety practices such as safety belts, air bags, etc.

Although there have been great improvements in quality of life of patients affected by SCI, it is worth mentioning that recent studies show less improvement in average life expectancy than had been postulated in the past (Strauss et al 2006, Shavelle et al., 2006, Pickett et al., 2006). A study looking at patients with SCI over the last 31 year period (Strauss et al 2006) reported that between 1973 and 2004, there was a 40% decline in mortality during the critical

first 2 years after injury. This study also found that during that same 31 year period, the reduction in mortality in the post 2 year period was not as statistically significant as would have been predicted.

1.3 Prognosis, classification and clinical assessment

The most important predictive factors for survival after traumatic SCI are patient age, the level of injury and neurologic grade (Claxton et al., 1998; Ditunno 1999). Greater mortality is reported in older patients with SCI. The degree of paralysis caused by injury to the spinal cord depends upon the level of injury to the spinal cord. The rule of thumb is that the higher the level of the lesion, the more severe the consequences. In the case of high cervical injury, patients usually require artificial respiration to stay alive. The following terminology has developed around the classification of SCI base on the extent of the injury:

- ***Tetraplegia*** (replaces the term quadriplegia) - Injury to the spinal cord in the cervical region, with associated loss of muscle strength in all 4 extremities.
- ***Paraplegia*** - Injury in the spinal cord in the thoracic, lumbar, or sacral segments, including the cauda equina and conus medullaris. Depending on the level affected, it may lead to paralysis of the lower extremities and part or all of the trunk muscles. Usually there is a loss of sensation in paralyzed limbs and other effects such as muscle spasms, pain and loss of bowel and bladder control.

The American Spinal Cord Injury Association (ASIA) developed international standards for examining and reporting the severity of SCI (Kirshblum et al., 2002; Ditunno et al., 1994; Maynard 1997). This system describes the level and extent of injury based on a systematic motor and sensory examination of neurologic function. The ASIA classification (ASIA, 2006) assesses motor function in ten muscle groups (arms, C5–T1; legs, L2–S1) and sensation (light touch and

pinprick) in 28 dermatomes (C2–S4/5) on both sides of the body. Table 1 represents ASIA’s five grades of spinal cord function, A to E, where E is normal. ASIA grade A describes individuals with the least remaining function. Such patients usually have little hope for recovery.

GRADE	DESCRIPTION
A	Complete: no sensory or motor function is preserved in sacral segments S4–S5
B	Incomplete: sensory but no motor function is preserved below the neurological level and extends through the sacral segments S4–S5
C	Incomplete: motor function is preserved below the neurological level and more than half of the key muscles below the neurological level have a muscle grade less than 3.
D	Incomplete: motor function is preserved below the neurological level and more than half of the key muscles below the neurological level have a muscle grade more than 3
E	Normal: motor and sensory function are normal

Table1.1: American spinal cord injury association (ASIA) impairment scale

The spinal cord conveys both afferent sensory and efferent motor information, so disruption of spinal cord function results not only in motor paralysis but also sensory and autonomic impairment distal to the injury (Schwab and Bartholdi, 1996; Hulsebosch, 2002). Sensory dysfunction contributes to the generation of pressure sores that, like bladder impairment, are a major source of morbidity and even mortality in those with spinal cord injury (Warren, 2010; Jackson et al., 2010; Silva et al., 2010; Evaraert et al., 2009; Nath et al., 1993). Nearly all spinal cord injuries damage both upper and lower motor neurons. To measure the contributions of each neuron to loss of function is important, since the prognosis for regeneration is very different for each. Segmental lower motor neurons are damaged or lost in the central grey matter

at the injury site and in several segments above and below the lesion, resulting in flaccid paralysis at the injury level. Variable injury to the surrounding white matter affects the long tracts, producing signs of upper motor-neuron damage below the level of the injury. In all lesions (apart from complete transections) a small doughnut-like rim of white matter remains at the injury site. This outer white matter allows some axons to remain intact, but many others cease to function because of segmental demyelination (Bunge et al., 1993). For a lesion to be confined to the grey matter (which contains neuronal cell bodies) and to spare the surrounding white matter is not unusual. Such a central cord lesion confines motor and sensory disturbances to areas innervated at that level (e.g. a C6 lesion affects the hands) without affecting functions by much below that level, such as gait and bowel or bladder function. By contrast, white-matter destruction at the same segment, even if grey matter is spared, renders a person tetraplegic and incontinent.

An important predictor of improved outcome is retention of sacral sensation (S4–S5), especially pinprick, 72 h to 1 week after injury (Oleson et al., 2005; Ditunno et al., 1999; Waters et al., 1995; Marino et al., 1999). In general, most individuals regain one level of motor function, and most recovery of function takes place in the first 6 months after injury, but can still arise several years later (Herbison et al., 1992; Waters et al., 1994; Stauffer, 1984).

1.4. Pathophysiology of SCI

The functional decline following SCI is attributed to both direct mechanical injury and secondary pathophysiological mechanisms that are induced by the initial trauma. These mechanisms initially involve widespread haemorrhage at the site of injury and necrosis of central nervous system (CNS) cellular components followed by a cascade of secondary events that lead

to further destruction including structures beyond the epicenter of the injury. In a nutshell, the pathological profile of traumatic spinal cord injury (SCI) comprises a series of changes that can be categorized into primary and secondary events.

1.4.1 Primary events

The primary events comprise the focal destruction of neural tissue and are a consequence of direct mechanical trauma to the spinal cord which includes traction and compression forces. This stage is characterized by direct compression of neural elements, damage to blood vessels, axons, neuronal cell membranes (by fractured and displaced bone fragments, disc material and ligaments) with consequential swelling of the spinal cord. Swelling of the spinal cord compresses blood vessels which gives rise to secondary ischemia eventually leading to spinal neurogenic shock which in turn leads to systemic hypotension and exacerbation of the ischemia. These initial events then instigate a progressive wave of secondary events, which via the activation of a stream of noxious mechanisms exacerbates the injury to the spinal cord leading to destruction of axonal tracts that were left intact by the initial trauma and hence causing a major impediment to functional recovery after SCI.

1.4.2 Secondary events:

In the past two decades, several important concepts have emerged to improve the understanding of secondary injury. Much of our understanding of these secondary events initiated by primary injury comes from knowledge contributed by studies of cerebral trauma and ischemia (Gentleman et al., 1995; Maxwell et al., 1997; McIntosh et al., 1998; Lee et al., 1999; Yanagawa et al., 2001; Hall et al., 2004). Prominent in the development of these delayed secondary mechanisms of damage is the interactive cascade that involves disturbances in energy balance, glutamate excitotoxicity, uncontrolled rises in intracellular Ca^{2+} and oxidative stress

(Juurlink and Paterson, 1998). These events are associated with neuronal somal and axonal loss that results in much of the disability following spinal cord injury. Many of these problems occur hours to days after the primary insult (Graham et al., 1995). Damaged cells, axons, and blood vessels release toxic chemicals that attack intact neighbouring cells. This highly disruptive process is known as excitotoxicity. A key contributor to this excitotoxicity process is glutamate. In the healthy spinal cord, the peripheral terminals of many axons release glutamate as a neurotransmitter which binds to receptors on target neurons, exciting these neurons. In injured spinal cords on the other hand, glutamate floods out of injured spinal neurons, axons, and astrocytes, overexciting neighbouring neurons which in turn release more glutamate (Liberto et al., 2004). The overexcited cells have an uncontrolled influx of calcium ions that trigger a series of destructive events, including production of free radicals and lipid peroxidation (Kaynar, M.Y., et al., 1998; Nishio et al., 1997). These highly reactive molecules can attack membranes and other cell components, killing healthy neurons (Kaynar, M.Y., et al., 1998; Nishio et al., 1997; Pedersen et al., 1999; Rauchova et al., 1995 McConnell et al., 1999; Springer et al., 1997; Picklo et al., 1999; Kristal et al., 1996). Excitotoxicity was thought to affect only neurons, but results from emerging studies suggest it also kills oligodendrocytes, the CNS's myelin-producing cells (Beattie et al., 2000; Liu et al., 1997; McDonald et al., 1998). Glutamate receptors known as AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors play a crucial role in oligodendrocyte injury (McDonald et al., 1998). This could explain why unsevered axons become demyelinated and therefore unable to conduct impulses after spinal-cord trauma.

It is clear that all these events interfere with axonal regeneration, resulting in axon disconnection, demyelination and cell death. In addition, there are longer term reactions such as reactive gliosis that also interfere with axon regeneration (von Euler et al., 1997; Frisen et al.,

1995; Ridet et al., 2000; Beattie et al., 2000; Profyris et al., 2004). The actions of astrocytes as well as numerous other cells in this response create an environment that is highly non-permissive to axonal regrowth (Hayashi et al., 2004; Fawcett and Asher, 1999; Profyris et al., 2004). Moreover, a more active form of cell death, somewhat akin to suicide, in the cord has also been documented in the past decade (Li et al., 1999; Shuman et al., 1997; Frei et al., 2000; Casha et al., 2001; Beattie et al., 2000). It has been observed that days or weeks after initial trauma, a wave of cell suicide, or apoptosis, might sweep through oligodendrocytes, affecting sites that are distant to the vicinity of primary injury (as many as four segments from the trauma site).

1.5. Molecular mechanisms in spinal cord injury

As can be implied from the previous paragraphs, several metabolic signalling pathways are implicated in the generation of post-traumatic cellular turbulence. The complex pathophysiology of SCI gives rise to activation as well as inhibition of numerous molecular mechanisms involved in secondary injury. The following paragraphs deal with mechanisms that are specifically tailored to address the objectives of this thesis and provide the precedence for administering quercetin as a possible therapeutic measure.

1.5.1 Oxidative Stress and inflammation

Oxidative stress can be defined as a condition in which the production of strong oxidants overrides the organism's scavenging capacity. Strong oxidants produced during oxidative stress include the superoxide anion, hydrogen peroxide, hydroxyl radicals, peroxiradicals, and dicarbonyls (Juurink, 2001). One major source of strong oxidants during oxidative stress is the superoxide anion, which can be produced during mitochondrial respiration. This substance can be dismutated to hydrogen peroxide by the enzyme superoxide dismutase (SOD). Hydrogen

peroxide, in the presence of transition metals such as Fe^{2+} or Cu^+ in turn can be converted to the hydroxyl radical (which is the most potent oxidant in biological systems), or lipid peroxy radicals (Gutteridge et al., 1992; Halliwell and Gutteridge, 1992; Juurlink and Paterson, 1998). Hydrogen peroxide can also give rise to pro-inflammatory isoprostanooids (Liu et al., 1998) and strong oxidants such as hydroxynonenal (Springer et al., 1997). Superoxide anions can react with thiols and de-esterify membrane lipids causing the release of arachidonic acid and initiating a self-perpetuating cascade creating a reservoir for the production of strong oxidants. They can also interact with other substances such as NO to produce a variety of strong oxidants including the hydroxyl radical, nitrogen dioxide and singlet oxygen (Juurlink, 2001). Other strong oxidants produced during oxidative stress are dicarbonyls (or α -oxo-aldehydes) that can give rise to advance glycation end products (Thornalley et al., 1993; Wells-Knecht et al., 1995) that are detrimental for tissues (Thornalley PJ, 1995).

The accumulation of strong oxidants and reactive oxygen species (ROS) during oxidative stress promotes excitotoxicity that leads to calcium overload and ATP depletion, which will eventually lead to cell death (Juurlink, 2001; Annunziato et al., 2003). It has been established that increases in intracellular calcium concentration lead to activation of proteases, phospholipases, endonucleases which degrade various cellular components including DNA and ultimately contribute to cell death (Cantoni et al., 1989).

Oxidative stress can also promote the activation of transcriptional factor complexes such as nuclear factor kappa B (NF κ B) and AP-1 through activation of c-jun N-terminal kinase (JNK) (Ip and Davis 1998). Activation of NF κ B results in pro-inflammatory gene expression (Christman et al., 2000; Baeuerle and Henkel, 1994). A major consequence of pro-inflammatory gene expression is the increased infiltration of activated leukocytes, particularly neutrophil and

macrophages. These activated leukocytes in turn contribute to maintaining the vicious interactive excitotoxic cascade.

1.5.1.1 Oxidative Stress and inflammation following traumatic SCI

Much of the secondary damage in post-traumatic SCI injury is driven by oxidative stress and the accompanying inflammation (Juurlink and Paterson, 1998, Halliwell et al., 1992; Anderson et al., 1993; Amar and levy, 1999; Tator and Fehlings, 1991; Tator et al., 1997, Panter et al., 1992; Christmas et al., 2000; Kirshblum et al., 2002; Joshi and Fehlings, 2002; Hall et al., 2004). Trauma to the spinal cord causes free radical formation and lipid peroxidation hence leading to oxidative stress and inflammation (Juurlink and Paterson, 1998; Kaynar et al., 1998; Nishio et al., 1997). It has been demonstrated that ROS overproduction can directly cause death of immature cultured cortical neurons (Ratan et al., 1994) and induce DNA damage as well as an inflammatory state (Juurlink and Paterson, 1998).

Just like any part of the CNS, the spinal cord is particularly vulnerable to oxidative stress because of its high oxygen consumption coupled with a low oxidant scavenging capacity as well as a high percentage of polyunsaturated fatty acids that can be subjected to lipid peroxidation by free radicals. Peroxidation of membrane polyunsaturated lipids interferes with membrane function resulting in a number of disturbances in cellular function such as the regulation of iron homeostasis. This would inevitably lead to increased consumption of ATP that would further enhance the formation of free radicals, (Christman et al., 2000). Breakdown products of lipid peroxidation include 4-hydroxyalkenals that inhibit glucose transport, ion pump activities (Rauchova et al., 1995; McConnell et al., 1999), glutamate uptake (Springer et al., 1997), mitochondrial respiratory chain (Picklo et al., 1999) and activation of the mitochondrial permeability transition pore (Kristal et al., 1996), all events that would exacerbate the energy

crisis and excitotoxicity. Lipid peroxidation of the endothelial membrane also appears to play a role in edema formation (Nishio et al., 1997). Lipid peroxides also give rise to pro-inflammatory isoleukotrienes (Harrison and Murphy, 1995) and pro-inflammatory isoprostanooids (Liu T et al., 1998), many of which promote the inflammatory response. Evidence for pro-inflammatory gene expression following neurotrauma includes upregulation of enzymes such as inducible nitric oxide synthase (iNOS) (Wada et al., 1998), neuronal NOS (nNOS) (Rao et al., 1999), cyclooxygenase-2 (COX2) (Dash et al., 2000) and endothelium cell adhesion molecules, necessary for leukocyte infiltration (Carlos et al., 1997; McKeating et al., 1998). Preventing lipid peroxidation decreases these inflammatory lipid byproducts (Pratico et al., 1998).

1.5.2 Axonal Damage

Neurotrauma is known to cause diffuse axonal damage (Blight, 1988; Gentleman et al., 1995; Maxwell et al., 1997). The evidence suggests that axonal damage due to posttraumatic inflammation stems from impairment in axoplasmic transport (Povlishock et., al 1999). Factors that result in axoplasmic transport impairment include elevated intracellular Ca^{2+} (Juurink and Paterson 1998; Dawson et al., 1993; Tanaka et al., 1994; Povlishock et., al 1999). Indeed, there is evidence for calpain-mediated proteolysis following CNS trauma (Kampfl et al., 1997).

As early as 15 min after injury there exists periaxonal swelling with the myelin laminations peeling away from each other (Profyris et al., 2004). Occurring at the same time is myelin rupture, and by 24 h, axonal contents can be observed in the extracellular space. The release of toxic chemicals from these cells, including glutamate, promotes excitotoxicity associated with calcium overload and production of ROS (Graham et al., 1995; Kaynar, M.Y., et al., 1998; Nishio, S., et al., 1997) and destruction of intact neighbouring cells. Neuronal axoplasms undergo transition as well; they display a granular appearance with disarray of their

neurofilaments and in many axons there is an unusual abundance of intracellular organelles (Anthes et al., 1995). As time progresses, other pathologic features of injured axons, such as those of widespread demyelination and abortive growth cones, are increasingly observed (Profyris et al., 2004). In fact, by 24 h, a common phenomenon is the appearance of giant axons (greater than 40 μm), which exhibit a combination of all the above-mentioned pathological features (Anthes et al., 1995).

It is clear that the consequence of these disturbances results in axoplasmic transport impairment. One prominent feature of axoplasmic transport impairment is diffuse accumulation of β -APP (Gentleman et al., 1995). Beta-APP accumulation is seen within 2 hr of injury in human patients (McKenzie et al., 1996) and there is a correlation between extent of axonal swelling and survival time in human patients (Wilkinson et al., 1999).

These axonal changes are ultimately accompanied by Wallerian degeneration, which in rodents lasts for several months and in humans for years (David, 2002). Wallerian degeneration describes the withering of axonal segments separated from their neuronal soma. The process commences with the degeneration of separated fibers, which is accompanied by fragmentation of their associated myelin. It continues with the accumulation of resultant debris and culminates with the phagocytosis of this debris by macrophages and microglia. As the primary injury severs both ascending and descending axonal tracts, Wallerian degeneration is seen both rostral and caudal to the initial lesion (Quencer, 2002). Furthermore, the sites of degeneration have a strong correlation with areas of delayed apoptosis which includes glia cells and oligodendrocytes that have lost trophic support from severed axons (Warden et al., 2001).

1.5.3 Reactive gliosis

Similar to any other CNS injury, SCI initiates reactive gliosis. Reactive gliosis is characterized by an increase in immunocytochemically-detectable glial fibrillary acidic protein (GFAP) and a marked increase in GFAP can be seen following spinal cord compression injury (von Euler et al., 1997). One function of reactive gliosis is to clear up debris and create a barrier to prevent secondary injury mechanisms from spreading. Unfortunately, with this comes the burden of massive deposition of molecules that inhibit axonal regrowth (Fawcett and Asher, 1999); this includes debris from myelin and dead oligodendrocytes, as well as oligodendrocytes that survive the primary injury. The initial component is subsequently followed by the activation and migration of microglia, which is also accompanied by invasion of blood borne macrophages.

Reactive gliosis involves a complex interplay of glial (microglia and macroglia) and non-CNS cells. The initial stage of reactive gliosis involves the invasion of phagocytes (glial and non-CNS cells) at the site of primary injury. Glial involvement begins with “reactive” microglial cells, followed by a series of events that in turn leads to the activation of astrocytes (macroglia).

Microglia are CNS glial cells that differ phenotypically and functionally from macroglia (i.e. astrocytes and oligodendrocytes) and are closely similar to tissue-resident macrophages (Kim and Vellis, 2005; Rezaie and Male, 2002). These cells, first described by del Rio-Hortega, are estimated to comprise about 10% of all cells in the brain parenchyma (Yokoyama et al., 2004). Microglial cells are present in all regions of the mature CNS, but are found at different concentrations in various areas (Schwartz et al., 2006). Their main function likely is to act as first line of defence against pathological insults in the CNS (Kreutzberg, 1996). They are considered to be the most potent antigen presenting cell type in the CNS. Reactive microglia express major histocompatibility complex (MHC) class I and II on their surface, which are

specific antigen presenting molecules. They also express the co-stimulatory proteins CD40 and B7 as well as intercellular adhesion molecule-1 (ICAM-1) (Benveniste et al., 2001; Finsen et al., 1993). Like macrophages, microglia also sense many extracellular chemical messengers such as adenosine triphosphate (ATP), acetylcholine and noradrenaline that are produced in response to changes in the extracellular ionic milieu (Guillemin and Brew, 2004). Moreover, reactive microglia release a large variety of pro- and anti-inflammatory molecules including various interleukins, monocyte chemoattractant protein-1 (MCP-1) (Kim et al., 2005), tumor necrosis factor alpha (TNF- α) (Taylor et al., 2005), reactive oxygen species (ROS), nitric oxide (NO), prostanooids, and complement factors (Nakajima et al., 2001). Reactive microglia can be identified based on their immunostaining with Mac-1, ED1, OX18, OX42, OX6 and OX1 antibodies (Ling et al., 1991). Following SCI injury, quiescent ramified microglia transform into reactive microglia (Kreutzberg, 1996; Stence et al., 2001) and migrate toward the injury site (Dihne et al., 2001; Giordana et al., 1994). Response to injury by microglial/macrophage may include: (1) proliferation of ramified resident microglia, (2) migration from adjacent intact brain areas, (3) recruitment of monocytes from the blood, (4) or a combination of all these processes (Ladeby et al., 2005). The peak of microglial activation is usually attained by 48-72 hrs following acute insult (Banati, 2003).

The invasion of phagocytes into the glial scar is then followed by intrusion of oligodendrocyte precursors (Fawcett and Asher, 1999). Despite the inclination by CNS myelin to inhibit their differentiation, these oligodendrocyte precursors may potentially differentiate into mature oligodendrocytes at 2 weeks after injury, in areas where axons are void of myelin (McTigue et al., 2001; Miller, 1999). Concomitantly, meningeal cells from the CNS surface also migrate into the glial scar. These cells go on to re-form the disrupted glia limitans (normally

composed of astrocytes) that surrounds the CNS by making contact with astrocytes and walling off the primary lesion (Fawcett and Asher, 1999). Shortly thereafter, multipotential progenitor cells from the sub-ependyma of the central canal also invade the site of primary injury and spread throughout the cord (Takahashi et al., 2003).

The end point in the evolution of the glial scar is migration and proliferation of astrocytes (Fawcett and Asher, 1999; Hatten et al., 1991). Astrocytes are the most numerous non-neuronal cell types in the CNS and make up around 50% of all glial cell types. These cells embrace all cellular components throughout the CNS using their fine branching processes (Bignami et al., 1972). The main function of astrocytes is to provide essential support required for normal neuronal functioning, including homeostatic maintenance of extracellular ionic concentrations, as well as the clearance and release of extracellular glutamate (Mazzanti et al., 2001). In addition, the formation and maintenance of the blood-brain barrier (BBB) is dependent on astrocytes (Fawcett and Asher, 1999; Hatten et al., 1991). Astrocytes also play an important role in regulating neuronal function through the release of neurotrophic factors, which direct neuronal development, and contribute to the metabolism of neurotransmitters, as well as the regulation of extracellular pH and K^+ levels (Haydon, 2000). Moreover, astrocytes may play an active role in synaptic plasticity (Ullian et al. 2001). Based on their morphology, they can be classified into two main groups; namely, the fibrous astrocytes, (containing more glial fibrillary acidic protein (GFAP) intermediate filaments in their cytoplasm) which are located in white matter and have long slender cytoplasmic processes, and the protoplasmic astrocytes, (relatively poor in intermediate filaments) which reside in gray matter and have numerous short, highly branched cytoplasmic processes (Tsacopoulos and Magistretti, 1996). The fibrous astrocytes thus stain more intensely with antibodies to GFAP than do protoplasmic astrocytes (Wagner et al., 1993).

In response to SCI, astrocytes up-regulate their production of glial fibrillary acidic protein (GFAP), become hypertrophic and appose many of their processes via gap junctions to the processes of neighboring cells. Collectively, these changes are termed astrogliosis, and this response is a common hallmark of neural injury and diseases in which inflammation is a prominent component (Hatten et al., 1991). Depending on the injury condition, astrogliosis may have beneficial effects for promotion of neuronal survival by the production of growth factors such as neurotrophins that support neuronal growth, or detrimental for neuronal functional recovery by the formation of a glial scar. After injury, astrocytes are involved in the uptake of potentially harmful substances, such as excitatory amino acids and K^+ , and also release cytokines (Norenberg, 1994, 1996). Upon their infiltration, astrocytes isolate the site of primary injury by delineating the necrotic area and enveloping it with a dense glial lining and basal lamina. As astrocytes also fill the empty space produced by the primary lesion, they eventually form the bulk of the glial scar (Fawcett and Asher, 1999). The glial scar is not conducive for axon regeneration (Nieto-Sampedro, 1999; McGraw et al., 2001), not only because of the invading astrocytes, but also due to the tendency of the surrounding environment to promote production of extracellular inhibitory molecules that inhibit axonal regrowth (Fawcett and Asher, 1999). These molecules together with the rigid structure of the glial scar therefore impose both a molecular and mechanical barrier to axonal regrowth. One such molecule that is known to interfere with axon regeneration is chondroitin sulfate proteoglycan (CSPG) (Nieto-Sampedro, 1999; Lemons et al., 1999). In addition, disruption of the blood-spinal cord barrier (BSCB) caused by secondary injury may allow fibroblasts to migrate into the glial scar (Grimpe and Silver, 2002). At more chronic stages (about 3 weeks), another non-CNS cell, the Schwann cell which normally provide myelination to axons in the peripheral nervous system (PNS), may migrate into the lesion as

well. Interestingly, Schwann cells have the ability to remyelinate denuded CNS axons (Li et al., 1999).

1.5.4 Apoptosis and Necrosis

During the past decade, considerable progress has been made in understanding the process of cell death. Cell death occurs by at least two mechanisms: necrosis or apoptosis, which have distinct histological and biochemical features (Kerr et al., 1972; Wyllie et al., 1980). Necrosis is always a pathological process and the trigger for death (e.g. acute trauma and ischemia) is often the direct cause of the termination of the cell. Apoptosis by contrast, is induced by the activation of a cascade of events that causes the destruction of the cell. It is a tightly controlled process that is part of the normal physiological mechanism of cell elimination. However, it is well-known that many diseases are also characterized by dysregulation of apoptotic processes. In addition, the same type of insult such as acute trauma, ischemia, heat, and irradiation, can induce either necrosis or apoptosis or both at different times following injury. Typically, the preference of one mode of cell death over the other usually depends on the severity and duration of the insult as well as the idiosyncrasy of the target cell (Barros et al., 2001; Buja et al., 1993; Lieberthal et al., 1998; Beattie and Bresnahan, 2000; Martin LJ, 2001). Necrotic cell death usually occurs as a result of an abrupt severe injury whereas apoptosis is a slower process caused by less severe afflictions. For example, after an acute ischemic insult, necrosis will occur predominantly in the acute phase of the injury, in the most severely affected areas (i.e. the core of the lesion) whereas apoptosis will be mostly appreciated in the later stages, in areas that are not severely affected by the injury (i.e. at the penumbra where collateral blood flow reduces the degrees of injury). The consequences of apoptotic and necrotic cell death for the whole organism are fairly different. During necrosis, cytosolic constituents spill through the

damaged plasma membrane into the extracellular space and will provoke inflammation whereas in apoptosis, these products are safely isolated by membranes and then consumed by phagocytes (Kerr et al., 1972; Wyllie et al., 1980; Padanilam, 2003).

A great deal of the biological events that lead to both necrosis and apoptosis take place in secondary injury after acute SCI (Beattie et al., 2002). Initial events usually lead to necrosis of cellular components. In the presence of certain conditions, such as ischemia and excitotoxicity, apoptosis may become the emerging cell death pathway at the time when necrosis is currently the most predominant degenerative event (Zipfel et al., 2000). Since signalling pathways such as those involving death receptors, kinase cascades and mitochondria, participate in both processes it is possible that the organism can be able to switch between necrosis and apoptosis concurrently, by modulating these pathways (Pietenpol and Stewart, 2002). In addition, anti-apoptotic mechanisms (such as Bcl-2/Bcl-x proteins and heat shock proteins) are equally effective in protecting against apoptosis and necrosis (Pietenpol and Stewart, 2002).

1.5.4.1 Necrosis

Necrosis is typically defined as passive cell death where by tissues and cells die through unregulated processes of destruction of membranes and cytosolic organelles (Pradelli et al., 2010; Kerr et al., 1972; Padanilam, 2003). The histological hallmarks of necrosis are mitochondrial and nuclear swelling, dissolution and dispersion of organelles throughout the cytoplasm. This leads to rapid destruction of the plasma membrane, cytoplasmic structures and the nucleus with leakage of intracellular contents into the extracellular milieu, resulting in inflammatory reaction. As earlier mentioned, necrotic cell death usually follows an acute event and occurs in areas that are most severely affected by the abrupt biochemical collapse (Friedlander RM, 2003). The rapidity with which these events occur makes this type of cell death

very difficult to treat or prevent. This type of cell death is thought to be a consequence of adenosine triphosphate (ATP) depletion. Several ATP-dependent ion channels become ineffective, leading to loss of ion homeostasis, disruption of actin cytoskeleton, cell swelling, membrane blebbing and eventual collapse of the cell (Baros et al., 1999; Maeno et al., 2000; Padanilam, 2002).

Studies also suggest that in addition to the passive mechanisms, active mechanisms may also participate in the necrotic process (Proskuryakov et al 2003; Padanilam, 2003). Necrosis is a prominent mode of death in various neurodegenerative conditions and also occurs as consequence to ischemic injury in organs including brain and heart (Padanilam, 2003). In ischemia or hypoxic injury, energy depletion occurs by defective ATP production combined with rapid consumption of ATP by ion pumps as well as through hydrolysis and leakage. The initial event consists of an increased Na^+ influx and release of ATP due to membrane leakage. The increase in intracellular Na^+ level leads to the activation of Na^+/K^+ ATPase resulting in energy dissipation. As a consequence of ATP depletion, many organelles such as lysosomes, Golgi apparatus, endoplasmic reticulum and mitochondria undergo significant biochemical alterations leading to severe damage to these organelles. ATP depletion can activate non-selective calcium channels promoting calcium release from intracellular stores and hence an increase in intracellular calcium. Another consequence of cell injury is increased ROS production accompanied by inefficient scavenging which contributes in establishing the condition of oxidative stress (Juurink and Paterson, 1998). The accumulation of ROS is very damaging to cell structures including membrane phospholipids, proteins and DNA. Moreover, increases in cytosolic calcium levels in turn causes the activation of calcium dependent enzymes such as proteases, lipases and nucleases leading to further ATP depletion and disruption of intracellular

organelles. The mitochondrial permeability transition pore (MPTP) becomes more permeable allowing the production of more ROS and respiratory chain component such as cytochrome C, a pro-apoptotic protein (Crompton M, 1999; Kroemer et al., 1998). Therefore opening of the MPTP may lead to either necrosis or apoptosis or both. The level of ATP is one of the major factors that determine whether a cell undergoes necrosis or apoptosis.

1.5.4.1.1 Necrosis in SCI: Following traumatic SCI, as secondary injury events disseminate, a necrotic wave originates at the site of the injury and begins to propagate in a centripetal and rostro-caudal manner. This process, which is further propagated by the accompanying infarction, becomes irreversible by 8 hour and may spread up to two vertebral levels above and below the lesion. Blood flow to spinal cord is limited as a result of both primary events such as vascular damage to arterioles, capillaries and venules (Young, 2002) and secondary events such as vasospasm (Koyanagi et al., 1993), thrombosis and neurogenic shock. All these mechanisms then lead to bradycardia, hypotension, decreased peripheral resistance and decreased cardiac output (Guha and Tator, 1988). At the cellular level, this manifests as a loss of both oxidative phosphorylation and the glycolytic pathway. This ultimately cripples cellular energy production causing necrosis through ATP depletion. ATP depletion triggers a barrage of necrotic mechanisms, which include loss of cell membrane potential loss, increases in membrane permeability, release of lysosomal contents and activation of calcium-dependent auto-destructive enzymes. These enzymes include proteases, phospholipases, ATPases and endonucleases which degrade plasma membranes, the nucleus and cytoskeletal components (Kumar et al., 2004; Tator, 1995).

1.5.4.2 Apoptosis

Apoptosis, also known as programmed cell death, is a strictly regulated process responsible for the ordered removal of superfluous, aged, or damaged cells (Elmore 2007; Zhang et al., 2005; Kerr et al., 1972; Wyllie et al., 1980). Unlike necrosis, apoptosis is a much slower process involving the activation of a refined cascade of events. Histological characteristics of this process are cytoplasmic and nuclear shrinkage, chromatin margination and fragmentation, with breakdown of cell into multiple spherical bodies (apoptotic bodies) that retain membrane integrity (Elmore 2007; Zhang et al., 2005; Kerr et al., 1972; Wyllie et al., 1980). In contrast to necrosis, this typical confinement of cellular debris avoids inflammatory reactions (Kerr et al., 1972; Wyllie et al., 1980). Other features of apoptosis are a reduction in mitochondrial membrane potential, intracellular acidification, free radical generation and externalization of phosphatidylserine residues (Elmore 2007; Zhang et al., 2005). Apoptosis can be implicated in both normal (physiological apoptosis) and disease states (inappropriate apoptosis) (Elmore, 2007; Bellamy et al., 1995; Chalmers-redman et al., 1997; Freidlander, 2003). It is likely that all cells of the human body possess the intrinsic capacity of undergoing apoptosis (Kroemer et al., 1998). Apoptosis is involved in many important cellular processes, including normal cell turnover, the immune system, embryonic development and metamorphosis. Contrary to necrosis, which is led by severe damage to these organelles, only subtle perturbations are required to trigger the refined mechanisms of apoptosis. Apoptosis is implicated in cells exposed to stressful stimuli or damage allowing a multicellular organism to efficiently remove old, superfluous or damaged cells. It occurs continuously in proliferating tissues and counterbalances excessive cell proliferation during mitosis (Elmore, 2007; Bellamy et al., 1995; Chalmers-redman et al., 1997; Freidlander, 2003).

Disturbances in apoptosis regulation illustrate the importance of apoptosis for normal homeostasis. Inappropriate apoptosis may lead to either increased or decreased cell death. An abnormal resistance to apoptosis induction correlates with malformations, autoimmune diseases (due to the persistence of superfluous, self-specific immunocytes) or cancer (due to mutated cells). In contrast, enhanced apoptosis participates in acute pathologies (such as acute trauma, ischemic damage or infection by toxin-producing microorganisms) as well as in chronic diseases (neurodegenerative and neuromuscular diseases, AIDS). Apoptotic death is also an element of the lesion that appears after brain or spinal cord injury (Freidlander, 2003; Beattie et al., 2002).

The execution of apoptosis involves the activation of a number of latent cyteine-dependent aspartate specific proteases known as caspases (Elmore, 2007; Zhang et al., 2005). The activated enzymes cleave proteins within the cell leading to its destruction. The apoptotic cells are then rapidly cleared by phagocytic cells. Caspases can be categorized into upstream initiators (caspase 1, 2, 4, 5, 8, 9, 10, 11, 12, 13) and downstream executioners (caspase 3, 6, 7 and 14). The critical aspect of caspase-mediated cell death lies in the events regulating the activation of the initiator caspases.

Initiator caspases are activated by cell death signals (e.g. TNF α , TLR, and IL-1) (Pradelli et al., 2010; Donovan et al., 2004; MacFarlane M, 2003; Gallaher et., 2001; Ashkenazi and Dixit, 1999). They possess autocatalytic activity and are characterized by a long N-terminal prodomain that regulates their activation. These initiator caspases may be subclassified into those possessing the N-terminal caspase recruiting domain (CARD) and those with the N-terminal death effector domain (DED). Activation of these caspases requires specific binding of a regulator molecule to the CARD/DED domain. Caspase 2, 8, 9, and 10 are the primary initiators of apoptotic cascade while caspases 1, 4, 11, 12, and 13 are involved in cytokine production to

maintain the process (Pradelli et al., 2010; Donovan et al., 2004; MacFarlane M, 2003; Gallaher et., 2001; Ashkenazi and Dixit, 1999).

Executioner (downstream) caspases are characterized by a short N-terminal prodomain and are activated by the upstream caspases. They degrade a variety of cellular components leading to the events that bring about cell death. Caspase 3, 6, and 7 are effectors of apoptosis and caspase 14 is involved in cytokine production. Caspases use two main mechanisms to mediate cell death and these include the systematic destruction of critical cellular substrates (which are crucial and begins with DNA degradation) coupled with transcriptional up-regulation of the caspases (Pradelli et al., 2010; Donovan et al., 2004; MacFarlane M, 2003; Gallaher et., 2001; Ashkenazi and Dixit, 1999). Anti-apoptotic signalling pathways negatively regulate caspase activation through phosphorylation and down-regulation of their expression.

In addition to caspases, *mitochondrial events* also exert a major control over apoptotic cell death. Numerous physiological and some pathological stimuli trigger an increase in mitochondrial membrane permeability (Kroemer et al., 1998). The mitochondrial permeability transition pore (MPTP) complex can function as a sensor for stress and damage, as well as for certain signals connected to receptors. Under stressful conditions, the mitochondria can release apoptogenic factors (such as hydrogen peroxide, cytochrome C) through the outer membrane and dissipate the electrochemical gradient of the inner membrane (Crompton M, 1999). Taking on the duty as cytochrome C gatekeeper is the Bcl-2 family of proteins. These proteins are known to control the mitochondrial outer membrane permeability (Pradelli et al., 2010; Donovan et al., 2004; Martinou and Green, 2001) as well as regulate the sequestration of apoptotic scaffold proteins (Pradelli et al., 2010; Donovan et al., 2004; Adams and Cory, 2001). This family comprises both pro-apoptotic and anti-apoptotic members. A distinctive characteristic of

members of this family is their ability to bind to the outer mitochondrial membrane and to form dimers. Dimers formed with members that belong to the pro-apoptotic group (including Bax, Bid and Bad, Bak, Bcl-1S, Bim, Bik) will facilitate the release of cytochrome C, whereas those formed with the anti-apoptotic group (including, A1, Bcl-2, Bcl-w, Mcl-1 and BclxL) will block its release. Interestingly, the two groups spend the majority of their time forming heterodimers with one another to counteract each other's function (Hengartner, 2000). As a result, the relative abundance of pro- or anti-apoptotic Bcl-2 family members is decisive upon the cells fate (Donovan et al., 2004; Casaccia-Bonnet, 2000). Moreover both members can be regulated through gene expression and phosphorylation

It is generally accepted that there are two principal ways of inducing apoptosis (Pradelli et al., 2010; Donovan et al., 2004; Kerr et al., 1972; Wyllie et al., 1980; Padanilam, 2003). The first involves the activation of death receptors in the plasma membrane (extrinsic induction) while the second occurs via the disruption of intracellular homeostasis (intrinsic induction). It was originally thought that cytoplasmic organelles were not involved in apoptosis (Nicholson DW, 1999). It is now clear that many organelles such as lysosomes, Golgi apparatus, endoplasmic reticulum and mitochondria also undergo significant biochemical alterations during apoptosis.

1.5.4.2.1 Intrinsic pathway: The mitochondrion is the most crucial component of the intrinsic pathway of apoptosis as it stores cytochrome C to prevent it from escaping into the cytosol (Donovan et al., 2004; Padanilam, 2003; Hengartner, 2000; Casaccia-Bonnet, 2000; Kroemer et al., 1998). When apoptosis is induced however, cytochrome C is released into the cytosol and complexes with Apaf-1 and pro-caspase-9, forming the apoptosome. This structure activates pro-caspase-9 to caspase-9 by proteolytic cleavage, which in turn activates the

executioner caspases, caspase-3, -6 and -7. The activated caspase-3 plays a crucial role amongst all these caspases as it spurs the cleavage of DNA, nuclear lamins, cytoskeletal components and proteins that inhibit apoptosis. In addition to cytochrome C, sequestered within the mitochondrion in an apoptosis-regulated manner is Smac/Diablo (Padanilam, 2003; Hengartner, 2000; Kroemer et al., 1998). Upon its release to the cytosol, Smac/Diablo enhances the progress of apoptosis by antagonizing the activities of cellular inhibitor of apoptosis proteins (cIAP), a protein group that blocks caspase activity (Hengartner, 2000). The apoptotic domino effect set off by cytochrome C is the reason why its release from the mitochondrion is tightly regulated by the Bcl-2 family of proteins. The relative predominance of pro-apoptotic Bcl-2 over the anti-apoptotic counterpart is the cause of apoptotic destruction of the cell (Casaccia- Bonnefil, 2000).

1.5.4.2.2 Extrinsic pathway: The extrinsic pathway of apoptosis is characterized by the binding of ligand to death receptors. The death receptor family, which includes TNFR, Fas, Décor Receptors (Pradelli et al., 2010; Donovan et al., 2004; MacFarlane M, 2003; Gallaher et., 2001; Ashkenazi and Dixit, 1999), are characterized by cysteine rich extracellular binding domains and a cytoplasmic death domain. Stimulation of these receptors causes receptor aggregation and conformational change in their death domains that triggers the recruitment of various adaptor proteins such as SODD, FADD, TRADD, RIP and DAXX (MacFarlane M, 2003; Gallaher et., 2001; Ashkenazi and Dixit, 1999). As a result of this interaction, there is the recruitment as well as activation of many pro-caspase-8 units (Hengartner, 2000)). The primary activity of caspase-8 is to cleave caspase-3 and thereby set off apoptosis. Caspase-8 however, can also initiate the intrinsic pathway of apoptosis by cleaving cytosolic Bid and causing it to translocate to the mitochondrial membrane (Hengartner, 2000).

1.5.4.2.3 Apoptosis in SCI: Apoptosis is an important mediator of secondary damage after SCI (Freidlander, 2003; Beattie et al., 2002). It occurs through at least two phases. In an initial phase, apoptosis accompanies necrosis in the degeneration of multiple cell types while at a later stage it is predominantly confined to white matter and involves oligodendrocytes and microglia (Beattie et al., 2000). The initial stage which occurs at the lesion center begins approximately 6 hours post injury and for several days thereafter the number of apoptotic cells in this region rises steadily. However, by 1 week the apoptotic count decreases and there is now an increase in apoptotic death away from the site of primary injury. This new apoptotic wave is predominantly localized in the white matter and can arise at large distances from the lesion centre (Crowe et al., 1997). Under certain conditions, such as ischemia and excitotoxicity, apoptosis may arise as the emerging cell death pathway at the time where necrosis is currently the most predominant degenerative event following SCI (Zipfel et al., 2000). Oligodendrocyte apoptosis is a widely dispersed phenomenon during SCI that leads to long-term and persistent demyelination (Crowe et al., 1997). Since each oligodendrocyte myelinates multiple axons, their death leads to denudement of many fibres that are left intact by the initial trauma. Consequently, this compromises the conductive ability of axons hence hampering the functional recovery of patients. Numerous intact demyelinated axons are observed after SCI (Bunge et al., 1993) and salvation of oligodendrocytes has the potential to be of great therapeutic value. Apoptosis of oligodendrocytes as a result of SCI arises due to their increased susceptibility to insult as a result of trophic support loss (Barres et al., 1992; Raff et al., 1993). Additionally, oligodendrocytes may also undergo apoptosis as a result of apoptotic cascades set off by activation of their surface death receptors (Casaccia-Bonnet, 2000). In response to these insults, oligodendrocytes and microglia employ components of both the intrinsic and extrinsic pathways of apoptosis.

1.5.4.3 Role of oxidative stress in necrosis and apoptosis

Oxidative stress is known to cause both necrosis and apoptosis (Kajiwara et al., 2001; Annunziato et al., 2003; Juurlink and Paterson, 1998; Beattie and Bresnahan, 2000; Denecker et al., 2001; Proskuryakov et al., 2003). The release of various mediators during oxidative stress may lead to the activation of signalling cascades involving death receptors. Death receptors are implicated in the regulation of necrosis and apoptosis (Davis RJ, 2000; Mochizuki et al., 2002; Gabai et al., 2000). In addition, the level of ROS may also influence the decision point between apoptosis and necrosis. The accumulation of high levels of ROS in the cell causes direct and irreversible damage of cellular components (such as proteins, lipids and DNA) leading to necrosis. Modest levels of ROS act as secondary messengers and regulatory molecules hence promoting apoptosis. In the mitochondria, ROS can cause direct damage to mitochondrial DNA, and enzymes complexes as well as the mitochondrial membrane. Increases in ROS and cytosolic calcium imposed by oxidative stress, cause inappropriate opening of the mitochondrial permeability transition pore (MPTP) which leads to deterioration of mitochondrial functioning (Paschen and Doutheil, 1999). This induces further ROS production by the mitochondria causing further damage. When the opening of the MPTP is rapid and profound, high amounts of ROS are produced resulting in necrosis whereas with slower opening of the MPTP the cell is able to conserve its redox state. However it has also been shown that low levels of ROS can induce necrosis (such as in caspase-independent TNF-induced necrosis where there are low levels of ROS) (De vos et al., 1998; Vercammen et al., 1998; Fiers et al 1999; Goossens et al., 1999). Other molecules that may be involved in deciding whether the cell undergoes necrosis or apoptosis include hydrogen peroxide and cytochrome C release from mitochondria (which binds to Apaf-1 to activate caspase 9 leading to apoptosis) (Denecker et al., 2001; Kajiwara et al., 2001), BIM

(associate with the microtubule-associated dynein motor complex and released in response to microtubule damage) and Noxa (may be turned on by p53 in response to DNA damage) (Vaux, 2002). Therefore a balance between negative and positive regulation of these signalling cascades is believed to be a fundamental factor in determining the fate of the cell.

1.5.5 MAPK kinase signalling pathways

Protein kinases are known to play a major role in modulating cellular responses. While some kinase pathways may lead to responses that promote cell survival, other responses may promote cell death. These responses mostly depend on the integrated sum of metabolic changes that result in integrated biological outputs. A major family of protein kinase pathways systems are the mitogen-activated kinase (MAPK) cascades (Fig. 2).

Mitogen-activated protein kinases are a family of Ser/Thr protein kinases widely conserved among eukaryotes and involved in many cellular programs such as cell proliferation, cell differentiation, cell movement and cell death. MAPK signaling cascades are organized hierarchically into three-tiered modules that sequentially activate each other by phosphorylation within a structurally conserved activation loop (for reviews see Kim and Choi 2010; Kaminska et al., 2009; Johnson and Lapadat, 2002; Wada and Penninger, 2004; Widmann et al., 1999; Kyriakis and Avruch, 2001; Wolf and Seger, 2002). MAP kinases (MAPKs) are regulated quite specifically by MAP kinase kinases (MAPKKs) whereas the MAP kinase kinases are regulated more or less specifically by MAP kinase kinase kinases (MAPKKKs). Pathway organization is mediated by scaffolding proteins (Engstrm et al., 2010; Kyriakis and Avruch, 2001) which also generate the specificity in signal transduction pathway (Engstrm et al., 2010; van Drogen, 2002). All these kinases are also regulated by phosphatases (Flach et al., 2010; shi et al., 2010; Haagenson and Wu, 2010; Saxena and Mustelin, 2000; Zhou et al., 2002).

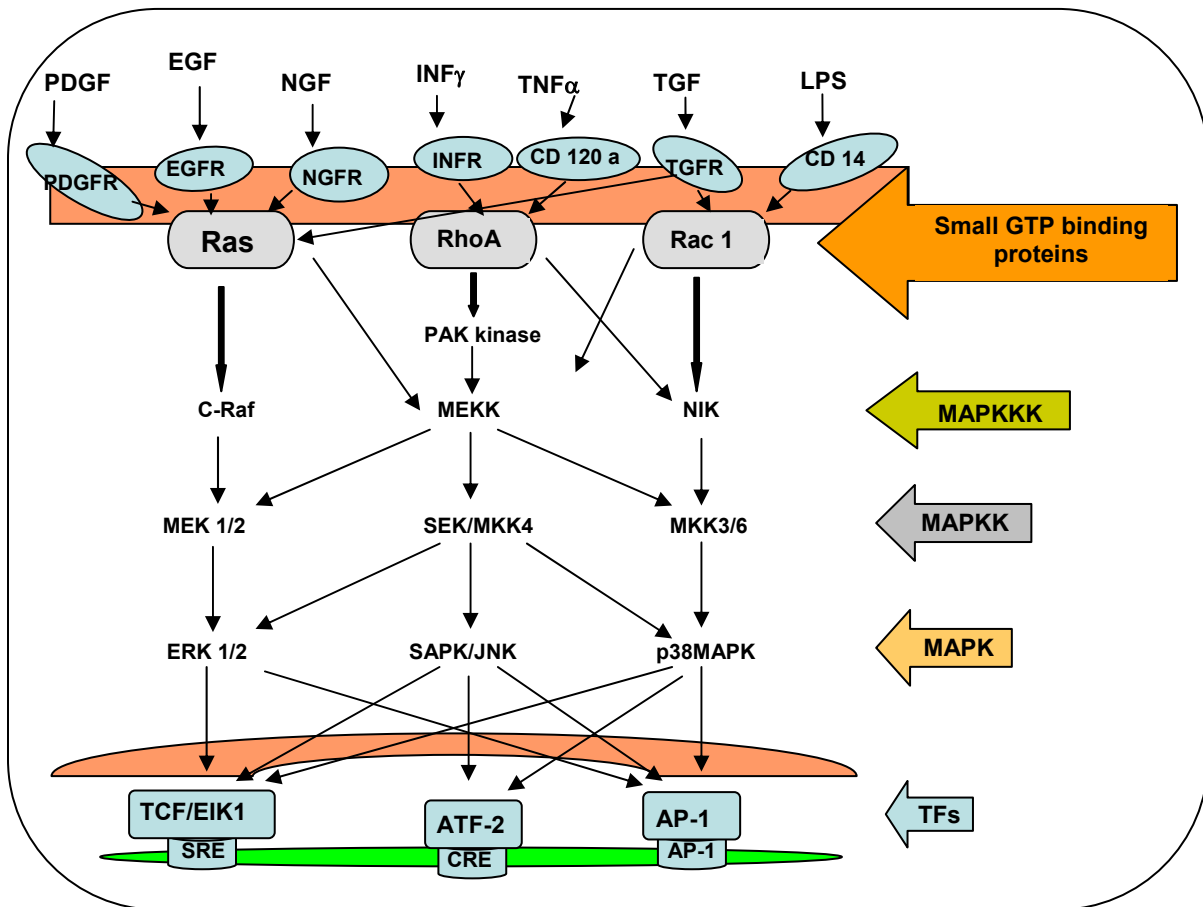


FIGURE 1.1: Activation of different MAPK signaling cascades by various extracellular stimuli. The ERK (Ras →Raf →MEK1/2 →ERK 1/2), JNK (MEKK →SEK/MKK →SAPK/JNK) and p38 (NIK1 →MKK3/6 →p38) cascades all contain the same series of three kinases. From these MAPK pathways, various transcription factors such as Elk1, ATF-2 (CREB), AP-1, NF-κB, Sp1 are activated and start the transcription of target genes.

Phosphorylated bottom tier MAPKs then translocate to the nucleus and phosphorylate their specific substrates (Kim and Choi 2010; Kaminska et al., 2009; Johnson and Lapadat, 2002; Wada and Penninger, 2004; Widmann et al., 1999; Kyriakis and Avruch, 2001; Wolf and Seger, 2002; Saxena and Mustelin, 2000).

The MAPKs include the extracellular signal regulated kinase 1 and 2 (ERK 1/2 or p42/44), p38 MAPK, and stress activated protein kinase (SAPK)/ c-jun N-terminal kinase (JNK) (Adler et al., 2000). MAPKs can be activated by a wide variety of different stimuli, but in

general, amongst these three MAP kinase cascades the ERK 1/2 are preferentially activated in response to growth factors while the SAPK/JNK and p38 MAP kinases are preferentially activated by cytotoxic stresses, ranging from X-ray/UV irradiation, heat/osmotic shock, and oxidative/nitrosative stress as well as by proinflammatory cytokines such as tumor necrosis factor α (TNF α) and interleukin-1 (IL-1). While the total level of these MAPKs remains constant, the level of the activated (phosphorylated) forms increases with the corresponding increases in stress signal. Therefore western blots analyses that are used to analyze activation levels of these kinases will show variations in the phosphorylated forms while the total MAPKs levels remain constant (Wu et al., 2009; Lal et al., 2008).

There are three SAPK/JNK genes each of which undergoes alternative splicing resulting in numerous isoforms (Kyriakis and Avuch 1999). SAPK/JNK, when active as a dimer, can translocate to the nucleus and regulate transcription through its effects on c-Jun, ATF-2 and other transcription factors (Kyriakis and Avuch 2001). Four isoforms of p38 MAP kinase, p38 α , β , γ (also known as ERK6 or SAPK3) and δ (also known as SAPK4) have been identified. Multiple ERK 1/2 (p44/42) MAPKs have been identified, including members of the Raf family, Mos and Tpl2/Cot (Meloche and Pouyssegur, 2007; Roberts and Der, 2007). MEK1 and MEK2 are the primary MAPKKs in this pathway (Rubinfeld and Seger, 2005; Murphy et al., 2006). MEK1 and MEK2 activate p44 and p42 through phosphorylation of activation loop residues Thr202/Tyr204 and Thr185/Tyr187, respectively. Several downstream targets of p44/42 have been identified, including p90RSK and the transcription factor Elk-1. ERK 1/2 are negatively regulated by a family of dual-specificity (Thr/Tyr) MAPK phosphatases, known as DUSPs or MKPs (Owens and Keyse, 2007), along with MEK inhibitors such as U0126 and PD98059. Although the ERK 1/2 pathway is commonly associated with cell survival, in some systems including the CNS, it

can also signal ischemic and stress-mediated cell death (Zhuang et al 2007; Kyriakis and Avruch, 2001). One of the crucial biological responses mediated by stress activated MAP kinase pathways appears to be the decision of cell fate by regulating apoptosis (Kim and Choi 2010; Kaminska et al., 2009; Sumbayev et al., 2005).

With reference to the role played by kinases, it is important to keep in mind that literature on the role of various protein kinases as playing protective or detrimental roles can be somewhat confusing. Much of this arises from lack of awareness of the contextual factor. It is becoming clear that local scaffolding proteins appear to generate much of the specificity in signal transduction pathways (Engstrm et al., 2010; Kyriakis and Avruch, 2001; van Drogen, 2002). Much of this confusion undoubtedly arises because we are not aware of all the contextual factors. Furthermore, this contextuality also allows cross-talk amongst pathways. For example, the MAP kinase kinase kinases, NIK and the MEKKs, can both activate I kappa B kinases, thereby activating the NFκB path (Baumann, 2000; O'Mahony, 2000; Zhao, 1999).

1.5.5.1 MAPK Cascades and Neurotoxicity

Increasing evidence suggest that the c-Jun N-terminal kinase (SAPK/JNK), the ERK 1/2 and p38 MAP kinase (p38) cascades are amongst the kinases that play an important role in promoting neurotoxicity (Zhuang et al 2007; Shen and Liu 2006; Schieven, G. L, 2005; Zarubin and Han, 2005; Widmann et al., 1999; Kyriakis and Avruch, 2001; Ip and Davis 1998; Christman et al., 2000; Baeuerle and Henkel, 1994; Wolf and Seger, 2002). JNK, p38 kinase and ERK5 are known to be involved in promoting inflammation (Kyriakis and Avruch, 2001). Activation of p38 MAP kinase and ERK 1/2 gives rise to much of the neurotoxic actions of microglia (Koistinaho and Koistinaho, 2002; Zhang et al., 1998; Bhat et al., 1998). Activation of JNK promotes the activation of transcriptional factor complexes such as nuclear factor kappa B

(NF κ B) and activator protein 1 (AP-1) (Ip and Davis 1998) that results in pro-inflammatory gene expression (Christman et al., 2000; Baeuerle and Henkel, 1994). Activation of the JNK pathway is required for the pro-inflammatory signaling of IL1 (Krause, 1998) and TNF α (De Cesaris, 1999; Verrecchia, 2003). Activation of JNK and p38 MAP kinase also promotes apoptosis in the CNS (Mielke and Herdegen, 2000; Nakahara et al., 1999). Stimulation of ASK1 (a MAP kinase kinase kinase) promotes prolonged activation of JNK and p38 kinase that results in apoptosis (Sumbayev et al., 2005; Tobiume K et al., 2001; Nakahara et al., 1999). Inhibition of MEK1/2 has been demonstrated to be neuroprotective (Wang X et al., 2003). ASK1 is activated following spinal cord injury as well as p38 and JNK (Nakahara et al., 1999).

ROS and other free radicals produced during oxidative can activate various MAPK pathways (McCubrey et al., 2006; Sumbayev et al 2005). Oxidative stress resulting from secondary injury can also trigger JNK (and NF κ B) pathways by inactivating protein phosphatases and thereby promoting kinase activities through disturbance of counterbalancing phosphatase activities (Morita K et al., 2001; Xu D et al., 2002). For example oxidants can inhibit protein phosphatase 5 (PP5) that in turns promotes apoptosis signalling kinase-1 (ASK1) activity (Tobiume K., et al 2001).

CHAPTER 2.0 THERAPEUTIC APPROACHES IN SPINAL CORD INJURY

2.1 Current clinical management of SCI

Despite enormous research on the quest for treatment of SCI, there is yet no universally accepted clinical management for the condition. Spinal cord injury predisposes patients to numerous complications. Therefore the care of such patients requires attention to multiple body systems, sensitivity to the effects of interventions on function and lifestyle, and a special vigilance because of the tendency of spinal cord injuries to mask problems.

The standard emergency management in North America involves stabilization of the vertebral column, maintenance of airways and arterial blood pressure and administration of high doses of the steroid methylprednisolone (MPO). The clinical care uses a combination of medical, surgical and rehabilitative approaches that may vary according to different states or centers. The medical and surgical approaches are aimed at limiting secondary injury while rehabilitation and long term follow-up have the key aim to prevent medical complications, which are the cause of re-admission in up to 30% of spinal cord injured patients (McDonald and Sadowsky, 2002; Warren, 2010; Jackson et al., 2010; Silva et al., 2010; Evaraert et al., 2009; Burns, 2007; Guihan et al., 2007; Deitrick et al., 2007; Ditunno and Formal 1994; Galloway 1997; Giannantoni et al., 2001; Esclarín de ruz et al., 2000). Measures to prevent complications must be implemented from the first day of the injury. Some of these measures include urinary drainage with a Foley catheter to prevent urinary retention (a major source of infection) as well as pressure relief for the skin to prevent pressure sores (another potential source of infection). These patients also require thromboembolism prophylaxis, measures to prevent gastric ulcers and bowel care to prevent colonic impaction (Kirshblum et al, 2002).

One of the few surgical options available comprises the early removal of damaging bone, disc and ligament fragments to decompress the spinal cord. However, the benefit of these surgical procedures still remains controversial (Heiden et al., 1975; Marshall et al., 1987; Vaccaro et al., 1997; Chen et al., 1998; Mirza et al., 1999) and clinical trials are needed to assess their effectiveness.

MPO was the first proven pharmacological treatment for SCI that was introduced in the 1990s and was the subject of a multicentre clinical study (Bracken et al., 1990, 1997, 1998). High dose MPO is still utilized in current management even though its use still remains a controversial treatment in many countries (Hulbert et al., 2000; Qian et al., 2000). Treatment with MPO has been associated with substantial risk of adverse side effects including increased gastric bleeding and wound infections (Galandiuk et al., 1993; Ducker et al., 1994; Short et al., 2000). Other experimental drugs such as naloxone, trilazad and monosialoganglioside sodium (GM-1 ganglioside) have been tested in multicenter clinical trials but have not attained primary endpoints (Bracken et al., 1997; Geisler et al., 1998).

2.2 Research Strategies for restoration of function in SCI:

During the course of the past two decades vast amounts of research have been conducted and are still being conducted to understand the injury mechanism in SCI. However, much still needs to be done to bring all the pieces together. Given the complexity of the injury mechanisms and knowing that the quest for a cure needs to take its course, it is important to adopt a very pragmatic approach to restorative treatment of traumatic SCI keeping in mind that improvement of the quality of life is an important factor (Anderson et al., 2008). A realistic treatment should aim at promoting meaningful recovery in a stepwise manner since it is clear that a cure will not

happen overnight. In this light it is therefore important to understand the hierarchy of needs of individuals. The hierarchy for quality-of-life improvements varies depending on level and severity of spinal cord injury, but in general, affected individuals prioritise bowel and bladder function, sexual function, hand function, and breathing.

Fortunately, the damaged spinal cord will not have to be completely rebuilt to improve quality of life. Small anatomical gains can produce disproportionate functional benefits. For example, fewer than 10% of functional long-tract connections are needed to enable some locomotory function (Blight, 1983). This level of connectivity often remains in the preserved doughnut-like outer rim of white matter after trauma, but axons in this rim might be non-functional because of faulty myelination (Bunge et al., 1993) or are impeded by the process of reactive gliosis. Therefore, limiting reactive gliosis and promoting remyelination of intact connections is one reasonable approach to improvement of function. Such limited restoration might not enable people with severe spinal cord injury to walk but it might improve bowel and bladder control, hand grasp, limb mobility, or breathing.

The management of spinal-cord injury will probably mean an orderly sequence of different interventions, each providing an incremental benefit (McDonald et al., 2002). This could be achieved by including different stages of treatment that would focus on separate targets. Moreover, some treatments could be implemented together while others could be done at different intervals. Some of the key targets to be attained during treatment include (1) limitation of cell death immediately after injury, (2) promotion of cell regeneration, and (3) replacement of lost cells.

2.2.1 Limitation of cell death

Limiting cell death immediately after injury may involve the use of drugs aimed at the prevention of progression of secondary injury mechanism. This therapeutic compound should have the ability to counteract the events that lead to the excitotoxicity process hence preventing oxidative stress and inflammation as well as necrotic and apoptotic cell death. Injured demyelinated axons could also recover in this situation since this will allow surviving oligodendrocytes to remyelinate axons. An environment that is conducive will also allow for lost oligodendrocytes to be replenished.

2.2.2 Promotion of cell regeneration

The strategy to promote cell regeneration would involve the use of substances that promote regeneration such as growth factors (e.g. NT-3, BDNF), as well as the use of substances that would guide axons to the proper targets or increase the expression of the host cell guidance molecules. In addition, this may include the implantation into the syringe of substances that may create bridges hence providing directional scaffolding that encourage axon growth (such as transplants of peripheral nerves or ensheathing glia that support axonal growth). Another, property of this compound could be the ability to block the release of substances that inhibit regeneration or the action of natural inhibitors of regeneration or downregulate the expression of inhibitory proteins.

2.2.3 Replacement of lost cells

The strategy to replace lost cells could involve the implantation of cells capable of generation of all cell types (e.g. embryonic stem cells and stem cells) or substances that induce undifferentiated cells to replace dead cells.

It is valuable to consider which repair strategies are most feasible. For example, limitation of secondary injury will be easier than restoration of damaged spinal tissue. A hierarchy of goals for the near, intermediate, and distant future can be established from results of studies in animals. The strategies should be achieved in animals before being attempted in man. For this reason the choice of the animal model should also be given great consideration. Crucial effort must be made not only in choosing the right species but also in understanding its pathology and demonstrating its close resemblance with that of the human pathology. This will avoid a situation of putting much effort into research that is ruined by the wrong model.

2.3. Choice of treatment compound

A number of factors must be given careful consideration before choosing a potential therapeutic compound for testing *in vivo*. Some of the most pertinent factors to take into consideration include the possibility of adverse effects, the knowledge of the pharmacokinetics and pathways influenced by the compound, as well as the cost and availability of the drug.

Adverse effects could result from the compound itself or its metabolites, or could be related to the route of administration of the drug. In this regards information from previous studies can be useful if available. Knowledge of adverse reactions may prompt us to take cautionary measures and gear us to make a decision based on whether the benefits of the drug outweigh its potential risks. On the other hand, the absence of previous side effects should not prompt us to rule out this possibility as differences in experimental setting may also have different biological impact on the organism.

Knowledge of the pharmacokinetics of a compound is essential in order to make a good estimate of the effective dose. Basically this involves acquisition of data on the compound's

absorption and elimination as well as time of maximal concentration and half-life. The absorption of a compound is influenced by its route of administration. In experimental settings, routes that guarantee standardised dosing (such as intravenous, intraperitoneal and subcutaneous) are usually preferred. However, an important aspect to consider for later transfer into clinical trials is the option of oral administration as this may also influence the patient's compliance. The interval of maximum plasma concentration and half-life of a compound are useful in determining the interval schedule for substance administration. Ideally, a compound should not take too long to reach its peak plasma concentration and should have a long half-life (12 h or above) to improve patient compliance. The knowledge of the route of elimination of a substance is also of crucial importance. If the eliminating organ is compromised this may lead to unexpected accumulation of the substance or its metabolites and this may have grave consequences to the organism as a whole.

The hypothesis of a study is usually inspired for the most part by information acquired from previous studies regarding some known pathways influenced by a compound. This may be helpful in planning the experimental model and may also give us some idea on what therapeutic effects to expect based on previous observations. However, it is important to keep in mind that a different experimental setting may influence pathways differently. Ideally, if the pathological pathway can be singled out precisely, the ideal compound would be one that can positively alter this pathway. However, in conditions that are known to involve numerous physiopathological pathways, the ideal compound could be one which has the ability to influence numerous pathways. Since none of the numerous physiopathological mechanisms that cause secondary damage in SCI (such as ischemia, hemorrhagic necrosis, excitotoxicity, oxidative stress, inflammation, reactive gliosis and apoptosis) have been singled out as the pertinent contributing

factor, it is meaningful to use a compound such as quercetin that has the ability to affect numerous of these pathways.

My research exploits the strategy of minimizing the evolution of secondary damage at a chronic phase following SCI. The choice of the flavonoid quercetin as a treatment compound is guided not only by its ability to interfere with numerous of the processes that are known to be involved in secondary injury, but also by its availability and wide therapeutic window.

2.3.1 Rationale for delayed administration of quercetin

Research in our lab has directed great effort to explore the therapeutic potentials of quercetin, following SCI injury in male Wistar rats. Studies in the lab have shown that the prompt administration of quercetin starting 1 hr post-injury at a dose of 25 $\mu\text{mol/kg}$ weight every 12 hr for 4 days gives rise to a significantly better locomotor functional recovery in the quercetin treated animals than the saline controls (Schültke et al 2003; Schültke et al., 2010).

This project examines the effects of quercetin after chronic SCI in male Wistar rats. Pilot studies that prompted the initiation of this thesis work had shown improvement of locomotor function in spinal cord injured rats when quercetin was administered at 14 days after traumatic SCI at a dose of 75 $\mu\text{mol/kg}$ weight every 12 hr for 3 weeks. Histological examination of spinal cord tissues from untreated animals at 2 weeks post injury showed that a significant proportion of axons were still intact although there appeared to be a paucity of support cells.

These observations suggested that, what quercetin treatment at 2 weeks appears to do is to allow survival of neurons and supporting cells that normally would die somewhere between 2 and 6 weeks after injury. The decision to delay treatment could have implications in the treatment of chronic SCI, which is an accepted reality in those suffering from this condition.

2.3.3 Flavonoids

Flavonoids, or bioflavonoids, are a ubiquitous group of substances belonging to the large family of polyphenols that are present in most plants, concentrating in seeds, fruit skin or peel, bark, and flowers (Middleton and Kandaswami, 1993; Noroozi et al., 2000; Lamson et al., 2000; Rahman et al., 2006; Desch et al 2010). A great number of plant medicines contain flavonoids, which have been reported by many authors as having antibacterial, anti oxidant, anti-inflammatory, antiallergic, antimutagenic, antiviral, antineoplastic, anti-thrombotic, and vasodilatory actions (Cook 1996; Avila et al., 1994; Ferrandina et al., 1998; Kang et al., 1999; Caltagirone et al., 2000; Mahmoud et al., 2000; Yang et al., 2001; Beecher, 2003; Rahman et al., 2006; Desch et al 2010). However, much of the molecular mechanisms behind the biological effects of these compounds still remain to be clearly understood.

Polyphenolic compounds in general, are characterized as containing two or more aromatic rings, each bearing at least one aromatic hydroxyl and connected with a carbon bridge (Beecher, 2003). For flavonoids the carbon bridge consists of three carbons that combine with an oxygen and two carbons of one of the aromatic rings (A) to form a third 6-member ring (C) (Figure 2.1). The connection of the B ring to the C ring as well as the oxidation state and functional groups of the C ring may differ among subclasses. Briefly, the structural components common to all flavonoids is the possession of 15 carbon atoms; two benzene rings (A and B) on either side of a 3-carbon ring (Figure 2.1). Multiple combinations of hydroxyl groups, sugars, oxygen, and methyl groups attached to these structures create the various subclasses of flavonoids. Over 4,000 different flavonoids have been identified within the major flavonoid

classes, which include flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones (Cook, 1996; Lamson et al., 2000; Rahman et al 2006).

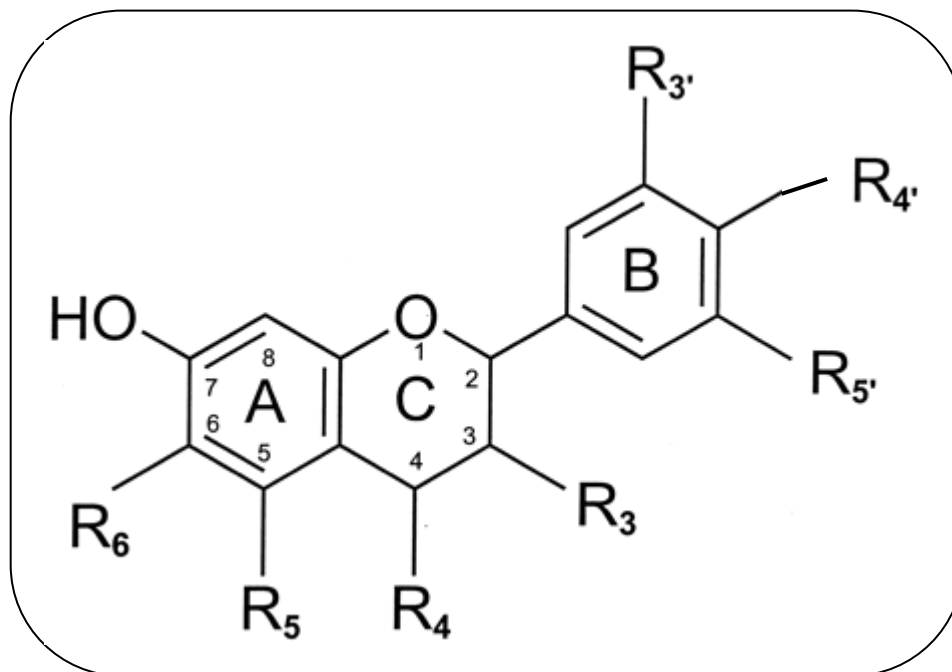


FIGURE 2.1: Basic chemical structure of flavonoids (adapted from Beecher, 2003). Common to these compounds include two benzene rings (A and B) on either side of a 3-carbon ring (C). Multiple combinations of hydroxyl groups, sugars, oxygen and methyl groups attached to these structures create the various classes of flavonoids.

Flavonoids have been shown in a number of studies to be potent antioxidants, capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxy radicals (Dugas, 2000; Duthie, 1997; Saija, 1995). Epidemiological evidence has shown that dietary intake of flavonols and flavones are inversely associated with coronary heart disease. It has been hypothesized that the ability of flavonoids to inhibit lipid peroxidation, demonstrated both *in vitro* and in various animal models, might, at least partially, account for this association (Hertog et al. 1993; Cook 1996; Schültke et al. 2003; Schültke et al 2010).

Flavonoids are absorbed from the gastrointestinal tracts of humans and animals and are excreted either unchanged or as flavonoid metabolites in the urine and feces.

2.3.3 Reasons for choice of quercetin as a therapeutic Agent

The flavonoid quercetin (3, 3', 4', 5, 7,-pentahydroxyflavone) has been widely studied for its important anti-inflammatory and anti-oxidant and anti-apoptotic properties (Huk, 1992; Cotellet, 1996; Siaji, 1995; Ferrandiz, 1991; Middleton and Kandaswami, 1992; Middleton et al., 2000; 1992; Pelzer, 1998; Lamson et al., 2000; Rahman et al., 2006; Mullen et al., 2006; Graf et al., 2006; Moon et al 2008). Quercetin is the major flavonoid in the human diet particularly abundant in fruits and vegetables, with the richest sources of quercetin being onions, apples, tea and red wine. It constitutes an important ingredient in Ginkgo biloba (Kleijnen and Knipschild, 1992) and some foods, such as wine and tea, may contain a concentration of quercetin of up to 150 μM (Goldberg et al., 1996). Estimated dietary intakes of quercetin range between 23-30 mg/day in European countries and the USA (Noroozi et al., 2000; Lamson et al 2000, Moon et al., 2008).

A lot of the biological effects of quercetin are attributed to its molecular structure, particularly the presence of an oxy-group at position 4 in the B ring and a double bond between carbon atoms 2 and 3 of the C ring and a hydroxyl group at position 3 of the C ring (Fig 2.2). This structural feature is known to be suitable for free radical scavenging (Theoharides et al, 2001) and is therefore believed to be a contributing factor for quercetin's potent antioxidant activity.

The toxicity of quercetin has been studied both *in vitro* and *in vivo* (Hollman, 1995; Ferry et al., 1996; Hollman, 1997; Hollman, 1999; Sestili et al 1998; Lamson et al., 2000; Khaled et., al 2003; Rahman et al., 2006; Mullen et al., 2006; Graf et al., 2006; Moon et al., 2008).

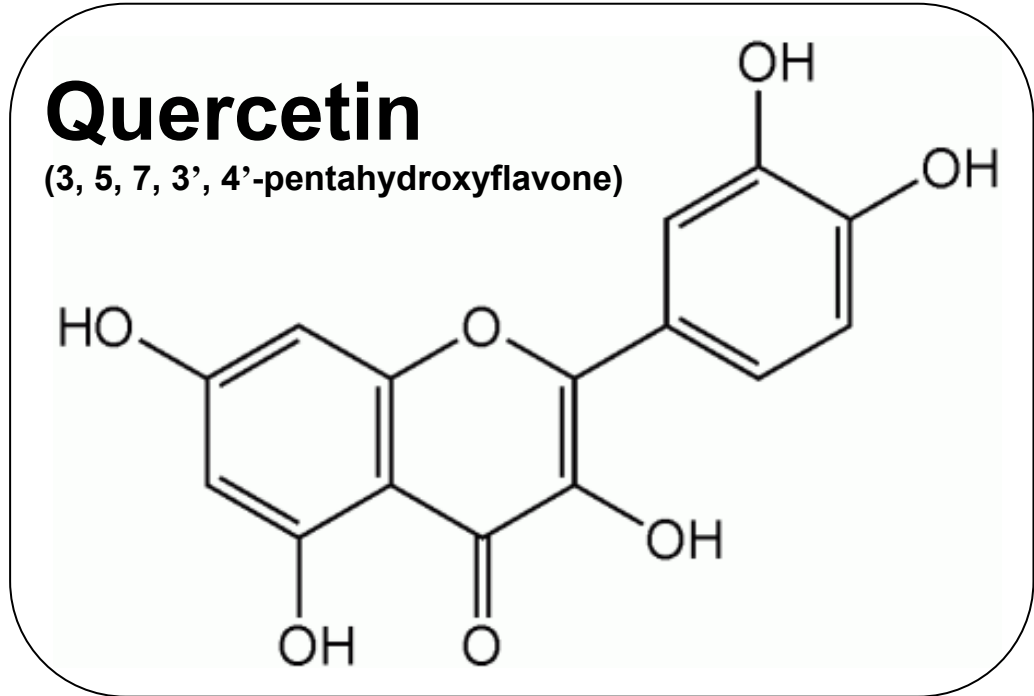


FIGURE 2.2: Structure of the flavonoid quercetin (3, 3', 4', 5, 7,-pentahydroxyflavone).

In rat studies, intravenous or oral administration of up to 56 mg/kg weight of quercetin did not produce any adverse effects (Khaled et al., 2003). In human studies, patients were safely administered a single i.v. bolus dose of up to 100 mg of quercetin (Ferry et al., 1996; Lamson et al., 2000; Moon et al., 2008). The peak plasma concentrations for quercetin have been reported between 0.7-7 hrs (Ferry et al., 1996; Lamson et al., 2000; Khaled et al., 2003; Mullen et al., 2006; Moon et al., 2008). Peak plasma levels of 225 ng/ml of quercetin were reached in patients after a 64 mg quercetin aglycone rich onion meal (Lamson et al., 2000). A single i.v bolus dose in humans of 100 mg quercetin led to a serum peak concentration of up to 4.1 mcg/ml of quercetin (Ferry et al., 1996; Lamson et al., 2000; Moon et al., 2008).

When recovered from dietary sources the disposition of quercetin in humans primarily depends on the sugar moiety (Moon et al., 2008; Mullen et al., 2006; Graefe et al., 2001;

Hollman 2001). About 25% of an ingested dose of quercetin is absorbed from the small intestine and is transported to the liver via the portal circulation, where it undergoes significant first pass metabolism (Olthof et al 2000; Williams et al, 2004; Rahman et al., 2006; Moon et al., 2008). Quercetin is extensively metabolized in the human liver and by the colonic microflora (Olthof et al 2000; Lamson et al 2000, Mullen et al., 2006; Rahman et al., 2006; Moon et al., 2008). Quercetin is strongly bound to albumin in the plasma. Quercetin and its metabolites are distributed from the liver to various tissues in the body. One of the most common metabolites of quercetin is isorhamnetin (3'-methoxyquercetin) (Manach et al. 1998; Morand et al. 1998). Since quercetin is quickly metabolized in the liver, it is possible that routes that avoid the first-past effect (such as the i.p and i.v routes) would be the desirable route to adopt. However, in a clinical setting, this route choice may affect patient compliance making the oral route more appropriate.

Pharmacokinetic studies on quercetin have not been very consistent regarding the elimination half life of quercetin. The half-life of various forms of quercetin have been reported to range between 5-25 hrs (Lamson et al 2000; Williams et al., 2004, Rahman et al 2006; Mullen et al., 2006; Moon et al 2008). However, in envisaging a possible clinical extrapolation of this study, it was a reasonable to choose the 12 h interval for quercetin administration for this study. This interval has also been adopted previously in our lab with positive findings (Schültke et al., 2003; Schültke et al., 2010)

To summarize, some of the pertinent reasons for choice of quercetin as therapeutic compound include the following:

1) Quercetin is an efficient chelator of Fe^{2+} (Morel, 1993) and possesses a more efficient iron (Fe^{2+}) chelating property compared to other chelating substances such as 2,2'-dipyridyl (Fe^{3+}) (Horky et al., 1998). Quercetin has been shown to prevent Fe^{2+} -mediated conversion of

hydrogen peroxide to the hydroxyl radical (Cheng and Breen 2000). This is likely the mechanism whereby quercetin has been shown to prevent hydrogen peroxide-mediated DNA strand breaks (Duthie, 1997).

2) It is present in many components of our diet and plasma levels may approach 1 μM following a quercetin rich diet (Hollman, 1995; Hollman, 1997; Hollman, 1999; Lamson et al., 2000). Humans have evolved with quercetin as part of their diet; therefore, quercetin is well tolerated. Therapeutic effects of quercetin have been shown when administered at a dose of 5 $\mu\text{moles/kg}$ body weights in acute traumatic SCI in rats (Schültke et al 2003; Schültke et al., 2010). Quercetin supplementation of up to 3 mmoles/day (i.e., ~ 50 $\mu\text{moles/kg}$ body weight) in humans is well tolerated (Conquer, 1998; Lamson et al., 2000; Moon et al., 2008).

3) It is readily absorbed across the gut and can attain micromolar levels in the plasma with the appropriate diet (Hollman, 1996; Moon, 2000; Lamson et al., 2000; Moon et al 2008).

4) Quercetin can minimize oxidative stress and accompanying inflammation as well as necrosis and apoptosis through a variety of other mechanisms (Theoharides et al., 2001; Moon et al., 2008). Quercetin is 7 times better as a scavenger of peroxy radicals than the vitamin E analogue trolox (Dugas, 2000; Lamson et al 2000). In addition to scavenging the lipid peroxy radical, quercetin has been shown to directly scavenge superoxide anion, the hydroxyl radical (Saija, 1995), singlet oxygen (Tournaire, 1993) and peroxy nitrite (Haenen, 1997).

5) Quercetin is an inducer of phase 2 protein genes (Valerio, 2001). Phase 2 proteins are involved in many of central anti-oxidant systems, (Juurlink, 2001). Quercetin is known to inhibit pro-inflammatory gene expression in several model systems: in a rat model of ureteral obstruction (Jones, 2000) and following $\text{TNF}\alpha$ administration to endothelial cells (Gerritsen, 1995). Furthermore, quercetin has been demonstrated to inhibit the activity of myeloperoxidase

(Schültke et al 2010; Schültke et al., 2003; Middleton, 1992) (a major means by which neutrophils exert their inflammatory function), xanthine oxidase (Sanhueza, 1992), 5-lipoxygenase and cyclooxygenase (Laughton, 1991).

6) Quercetin has also been shown to inhibit a variety of protein kinases (Davies et al., 2000; Rahman et al 2006). It is known to inhibit the activation of JNK by the lipid breakdown product 4-hydroxynonenal (Uchida, K., et al 1999). Quercetin inhibits the activity of I kappa kinase α and β (Peet and Li., 1999); these kinases are involved in phosphorylating I κ B, thereby activating the NF κ B pathway. Hence, this may be another pathway whereby quercetin inhibits inflammation. In addition, quercetin has been shown to inhibit hydrogen peroxide (H₂O₂)-induced apoptosis in glomerular mesangial cells via the inhibition of JNK/activator protein (AP-1) pathway (Ishikawa et al., 2000). Hence, some of the neuroprotective effects of quercetin may be mediated by inhibiting kinases that promote cell death. It may also be possible that quercetin alters the balance of kinase activities that promote cell death for kinase activities that are associated with cell survival.

2.4 Choice of Model

A careful choice of the appropriate model is crucial for the validation of any experimental study that needs to be extrapolated to human pathology. Cautious consideration should be given to known variations in the model and the actual human pathology. Issues such as differences in pathways and species should be taken into account as well as limitations regarding testing of functional outcomes in the animal model. We should have encouraging data to suggest that functional improvement in the animal model would translate into functional valuable

improvement in human patients. Moreover, the model should be reproducible between different researchers.

While important details about specific pathways can be studied *in vitro* where the researcher has total control over the experimental environment we should keep in mind that this needs to be extrapolated to higher organisms. However, certain components such as severity of the injury need to be controlled to a certain extent to avoid uncontrollable diversity within the species. In addition, the genetic make-up of the species should vary as little as possible to humans; therefore a mammalian model would be more realistic. Even though big apes would be the ideal candidates, the high number of subjects needed and the financial restraints that would be incurred greatly limit this possibility. Rats are on the other hand are readily available and easy to handle even in large numbers. Moreover, there exist striking pathophysiological similarities between clinical SCI and rat experimental models of SCI (Tator, 1995). However, it is worth mentioning that there are also striking differences between the regulation of the secondary events after SCI between animal strains and species (Hausmann, 2003). Therefore, extrapolation from animal experimental data to human pathophysiology requires caution.

2.4.1 Transection versus non-transection models of SCI

To mimic the majority of mechanical events that lead to various forms of human SCI, several experimental models have been developed (Beattie et al., 2002). A major distinction between SCI models is based on the initial presence or absence of continuity of spinal cord elements following the insult. In contusion and compression models of SCI the initial continuity of the spinal cord elements is largely conserved whereas in partial and complete transection models this continuity is lost.

Transection models: Transection models can be further subgrouped into complete and partial transections. Contrary to spinal cord injury caused by contusion or compression, only limited spread of rostro-caudal secondary injury has been observed in transection models (Dushart and Schwab, 1994). This limited spread of injury after transection likely occurs by Wallerian degeneration (Beattie et al, 2002; Hausmann, 2003). Models of complete transection have the advantage of a greater level of uniformity, as compared to models of contusion and compression, where minor variations in the localization of the injury can cause significant variation in the degree of recovery. Effectiveness of interventions regarding to both axonal regeneration and functional recovery are to some extent easier to evaluate with transection models (Kwon and Tetzlaff, 2001). However a major difference with the human model of transection is that in animals the dura needs to be open to create the injury. Hence intradural pressure that develops from edema does not reach the high levels attained in humans with intact dura. Partial transactions have the advantage of allowing the researcher to selectively injure specific tracts and use the uninjured side as control. However they have the disadvantage of not being able to ensure the exactness of the transection. Moreover functional compensation of the uninjured side may mistakenly enhance the perceived recovery of the injured side.

Non-transection models: In the clinical setting of acute traumatic spinal cord injury, a high percentage of patients do not present with complete transection of the spinal cord, but rather with a blunt injury containing elements of contusion, compression and possibly partial transection (Kakulas, 1984). Complete functional loss may only develop later as a result of secondary injury mechanisms (Fehlings and Tator, 1988; Tator and Rowed, 1979). Therefore, compression or contusion models of spinal cord injury more closely simulate the clinical situation seen in the majority of cases. A major difference with transection injury is that even

with severe injuries, a small peripheral rim of spared axons usually remains (Bresnahan et al., 1987; Basso et al., 1996; Kamencic et al., 2001). This observation made in animal models of spinal cord injury is paralleled by autopsy findings in human patients with neurologically complete spinal cord injury (Hayes and Kakulas, 1997). Those spared axons might be a good target for therapeutic intervention after SCI (Beattie and Bresnahan, 2000; Kamencic et al., 2001).

The most commonly used SCI model is the contusion model (Tator, 1995 and Young, 2002). This model induces instantaneous mechanical deformation of the spinal cord by dropping either a weight (Noble and Wrathall, 1985), an impactor rod (Gruner, 1992) or an impounder with computer-guided assistance (Bresnahan et al., 1987). In contusion injuries the site of injury is characterized by the development of a central hemorrhagic necrosis, which spreads both radially and in a rostro-caudal direction and later develops into a cystic cavity with an irregular margin (Bresnahan et al., 1976 and 1991; Guizar-Sahagun et al., 1994; Zhang et al., 1997). These weight-drop models have a number of disadvantages, an important one of which is its ability to produce considerable variability in clinical outcome and in the pathology at the site of injury (Khan et al., 1985; Khan and Griebel, 1983). Furthermore it was noticed that weight drop models cause primarily posterior cord compression, while in the clinical situation of human spinal cord trauma, more often the circumferential type of spinal cord compression occurs.

The compression model is another model frequently employed in SCI research. In this model, injury is induced either by intraspinal extradural balloon compression (Tarlov, 1957) or by applying either a weight or an aneurysm clip to the spinal cord (Rivlin and Tator 1977; Tator and Fehlings, 1991). This model aims to add to that of the contusion model by replicating the persistence of cord compression that is commonly observed in human SCI (Tator, 1995).

The decision to choose one model over another is greatly influenced by the research objectives and the hypothesis to be tested. My studies were done on a rat compression SCI model (described in materials and methods chapter), which is justifiably extrapolated to the human form of injury.

2.5 Importance of studying chronic spinal cord injury

Until recently, even the most optimistic of doctors believed that improvement in SCI were possible only within 6 months after the injury. With the advent of modern research a number of cases of late neurologic recoveries following SCI have been reported in literature (McDonalds 2002; Kirshblum, 2004). This has radically changed the way we view this condition and also given hope for new avenues for research. Our understanding of the disease as we know has been challenged. We are called upon to include a patient's hierarchy of priority in our treatment strategies. Slight improvement could bring about dramatic changes in the patient's way of life. This could be a little difference like being able to have slight awareness of bladder function or minimal sensation of touch, etc. For all these reasons studying therapeutic interventions in chronic SCI could be of good relevance. Moreover, such research would provide more insight to some of the mechanisms that occur not only after SCI but other neurodegenerative diseases such as multiple sclerosis (MS).

CHAPTER 3.0

SPECIFIC AIM, HYPOTHESES, OBJECTIVES AND QUESTIONS ADDRESSED

3.1 Specific Aim

The aim of this research project is to investigate the mechanisms by which quercetin promotes cell survival in late spinal cord injury, particularly its influence on signaling pathways leading to apoptosis. The main mechanisms of action addressed include the effects of quercetin administration on inflammatory responses, apoptosis, tissue sparing and MAPK signaling pathways.

My research deals with delayed administration of quercetin following traumatic spinal cord injury. Pilot studies that prompted the initiation of my thesis work had shown over 50% of locomotor function improvement when quercetin was administered 14 days after SCI at a dose of 75 $\mu\text{mol/kg}$ weight every 12 hr for 3 weeks. On histological examination of animals that were untreated at 2 weeks it was observed that a significant proportion of axons were still intact although there appeared to be a paucity of support cells. Hence it was postulated that quercetin treatment at 2 weeks appears to allow survival of axons and supporting cells that normally would die somewhere between 2 and 6 weeks after injury.

These observations prompted the development of the following hypotheses and objectives:

3.2 Hypotheses

1. Quercetin promotes locomotor function recovery in chronic spinal cord injury and is associated with decreased cell damage and neural tissue sparing.
2. The protective effect of quercetin in spinal cord injury is associated with decreased cell apoptosis
3. Quercetin improves axon function and decreases excess inflammatory responses in SCI.
4. Quercetin down regulates phosphorylation of various MAPK kinases that are known to be implicated in oxidative stress, inflammation and apoptosis in SCI.

3.3 Objectives

1. Determine the effect of delayed quercetin administration on locomotor function recovery following traumatic SCI.
2. Determine the effect of quercetin on cell damage and neural tissue sparing.
3. Determine whether quercetin administration is associated with decreased cell apoptosis
4. Determine the effects of quercetin on axoplasmic transport and inflammatory responses
5. Determine whether the protective effects of quercetin correlate with down-regulation of MAP kinase cascades involved in inflammation and/or apoptosis

3.4 Questions addressed and experimental approaches

3.4.1 Question #1: Does delayed quercetin treatment promote locomotor functional recovery following traumatic SCI?

Approach: To determine whether delayed administration of quercetin would promote functional recovery after SCI, behaviour testing was done by two individuals blinded to the study. These tests included the BBB and the incline plane test which were done twice weekly starting the first day post surgery until the last day of the study period. At the end of the eight week study period, a weekly average score was obtained for each group. This was done by averaging the highest weekly scores from both observers of all animals in each group. The postoperative performance of the quercetin treated and saline control animals were compared by 2-way-analysis of variance to determine if there were statistically significant differences between treatments. A p value < 0.01 was considered statistically significant.

3.4.2 Question #2: Does quercetin treatment at 2 weeks after injury decrease cell damage and promote tissue sparing?

Approach: The question was addressed by comparing tissue sections from animals that were harvested at the eight weeks study endpoint. Spinal cord tissue was harvested (T4-T8) and tissue sections processed as explained in the materials and methods chapter (section 4.7.). Morphological studies were done on sections from the quercetin treated, saline control and sham animals stained with hematoxylin and eosin (H and E), luxol fast blue (LFB) and toluidine blue (TB) to assess the differences in the extent of cavity formation and preservation of cellular architecture in all treatment groups. White matter content was assessed and quantified using sections stained with luxol fast blue (see section 4.9.5). ANOVA was used to determine

statistically significant differences between the quercetin treated and saline control groups and the statistical significant difference was set at $p < 0.01$.

3.4.3 Question #3: Does quercetin treatment at 2 weeks after injury decrease cell apoptosis?

Approach: The question was addressed by analysing tissues section and tissue homogenates from the 3 experimental treatment groups (3 day and 2 week treatment regimen) that constitute the study. Quercetin treated, saline control and sham animals from each group ($n=6 \times 3$ per experimental group) were sacrificed at eight weeks post surgery and spinal cord tissue was harvested (T4-T8) and tissue sections processed as explained in the materials and methods chapter (section 4.7.). TUNEL staining was used to determine and measure DNA fragmentation as a marker of apoptosis. Immunocytochemical and western blot analyses using various markers of apoptosis (activated caspase 3, cleaved PARP) were done to compliment the TUNEL analysis. Western blot data and TUNEL images were quantified and analyzed as outlined in the next chapter (sections 4.8.3; 4.9.4; 4.10.2 and 4.10.3). ANOVA was used to determine statistically significant differences between the quercetin treated and saline control groups. A p value < 0.01 was considered statistically significant.

3.4.4 Question #4: To what extent does quercetin improve axon function and reduce inflammatory responses in SCI?

Approach: Tissue sections from quercetin-treated and saline control animals from all 3 experimental treatment groups were immuno-stained for beta amyloid precursor protein (β -APP) accumulation as an indicator of impaired axoplasmic transport (hence axon function). Sections were also immuno-stained for GFAP (as a marker for astrogliosis), ED1 and OX42 (as markers

for activated macrophages/microglia). Tissue homogenates from the same group of animals transcardially perfused with saline were processed for Western blot analyses using the same markers mentioned above. The intensity of Western blot band signal for each antigen in each group were analyzed and quantified using NIH Image-J software (see section 4.8.3). Statistical difference between treatments groups were analyzed using ANOVA. A p value < 0.01 was considered statistically significant.

3.4.5 Question #5: Does quercetin treatment after injury down-regulate MAP kinase pathways associated with neurotoxicity and apoptosis?

Approach: Tissues from the 3 experimental treatment groups harvested at the study end point were used for this study. Tissue sections from the quercetin treated and saline control groups of animals were immunostained for various phosphorylated MAP kinases. These included phospho-JNK, phospho-p44/42 and phospho-p38 MAP kinase. Tissue homogenate from the quercetin treated, saline control and sham group of animals transcardially perfused with saline were processed for Western blot analyses of phosphorylated kinases. The results of intensity of the band from Western blots were quantified using NIH Image-J software and analyzed as explained in the methods chapter. The values obtained for each animal in the two treatment groups were analyzed with ANOVA setting a statistically significant difference at $p < 0.01$.

4.1 Animal care

All experimental protocols were approved by the University of Saskatchewan Standing Committee on Animal Care and Supply in accordance with the policies established in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care. All efforts were made to minimize the number of animals used and their suffering.

Male Wistar rats weighing between 250 and 300 g obtained from Charles River Laboratories Inc (St Constant, PQ) were used for all experiments. The animals were acclimatized for a minimum of five days before initiation of the study. They were cared for and housed in a temperature regulated facility (22-24°C) with 12-hour light/dark cycles. The animals had access to food and drinking water *ad libitum* throughout the duration of the study.

4.2 Experimental Design:

A schematic representation of the experimental design for this study is shown in Fig. 4.1. For all experiments, the animals were acclimatized for handling and test situation for five minutes twice daily, for at least five days before spinal cord injury surgery. Following surgery, animals were subject to proper post surgical care and began weekly locomotor testing but did not receive any treatment regimen until two weeks after the surgery. Locomotor testing continued during treatment and throughout the duration of the studies. The sham animals underwent surgery for spinal laminectomy but no spinal cord injury was inflicted in them. As such they did not receive any treatment throughout the study. At the end of the study period, all animals were euthanized and tissue samples were collected for various histological and molecular studies.

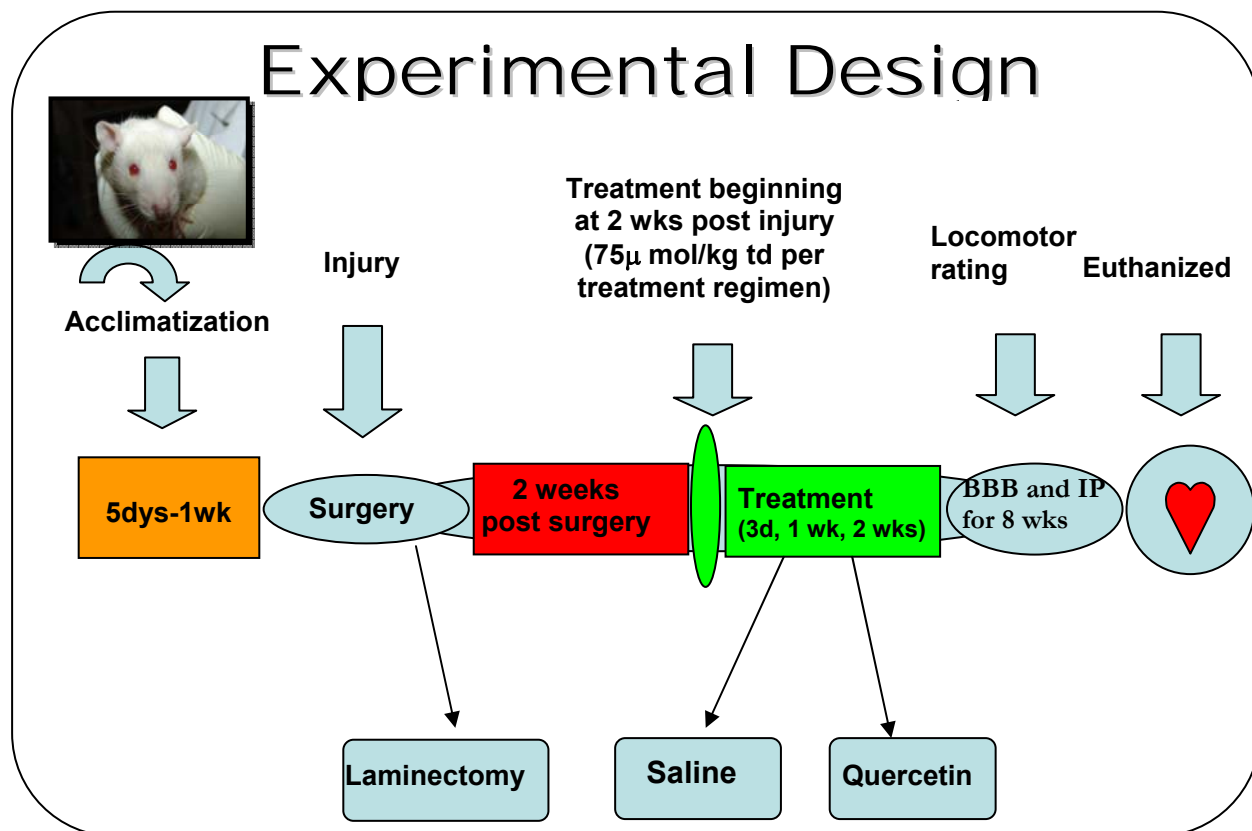


Fig 4.1: Schematic representation of the experimental design.

4.3 Surgery

For all experiments we used the spinal cord compression injury model first described by Rivlin and Tator (Rivlin and Tator 1977; Rivlin and Tator 1978). All surgical procedures were performed using the same protocols previously used in our laboratory (Schültke et al., 2003; Kamencic et al., 2001; Schültke et al., 2010). The procedures were all done under antiseptic conditions. Anaesthesia was induced with 5% halothane (Halothane® MTC Pharmaceuticals, Cambridge, ON, Canada) in medical grade oxygen (Praxair, Saskatoon, SK) and maintained between 1.5-2% halothane in oxygen at a flow rate of 1.5 liters/min delivered via a face mask. Surgery only began after it was ascertained that the animal was anaesthetized by applying grip

pressure to the paw and anesthesia was indicated by no paw withdrawal. The backs of the animals were shaved and prepared with 70% alcohol and chlorhexidine gluconate (Hibitane® Ayerst Laboratories, Montreal, Quebec Canada). All animals were pre-medicated with a subcutaneous injection of 0.05 mg/kg of buprenorphine hydrochloride (CDMV, St. Hyacinth Quebec) for postoperative analgesia. Animals were later administered five more tapered doses of the analgesic at 12 hr intervals.

To perform the surgery, a dorsal midline incision was made at the back of the animal using the spinous processes of thoracic vertebrae T4-T7 as landmarks. The underlying muscle was bluntly dissected and expanded, permitting access to the lamina of the spinal vertebrae. A laminectomy was then performed to expose the spinal cord and spinal cord injury was induced by compression with a 50 g force aneurysm clip (Kerr Loughheed clip, Walsh Manufacturing, Oakville, Ontario) to the mid-thoracic cord (T5/T6) for five seconds. This clip compression force induces a moderate to severe type SCI (Nashmi et al., 1997; Poon et al., 2007). This injury gives rise to permanent paraplegia and loss of bladder function for several weeks with animals never attaining a BBB (Basso, Beattie and Bresnahan) locomotor rating score above 5 in the absence of therapeutic intervention. A BBB score of 5 represents extensive movement of one joint and slight movements of two of the three hind limb joints (see Table 4.2).

The wound was then closed in anatomical layers by apposition of the underlying muscles followed by the subcutaneous layer and the skin. The skin was closed with mechanical staples (1Proximate® Plus MD, Ethicon Endo-Surgery, INC Cincinnati, OH USA). Staples were removed by day 5-7 post surgery. The injured animals were housed in individual cages for the first postoperative week and then caged in pairs until they were euthanized.

Surgery to the sham group of animals was limited to a laminectomy with no spinal cord compression. These animals all returned to preoperative performance within 24 hours after surgery. They all showed no loss of locomotor nor bladder functions.

4.4 Post surgical care

Scrupulous measures are taken to avoid infections and other complications related to the loss of sensation and immobility state (Warren, 2010; Jackson et al., 2010; Silva et al., 2010; Evaraert et al., 2010; Burns, 2007; Guihan et al., 2007; Deitrick et al., 2007; Ditunno and Formal 1994; Galloway 1997; Giannantoni et al., 2001; Esclarín de ruz et al., 2000). A close urological follow-up is an important part of these measures and includes regular emptying of the bladder to avoid urinary tract infections. Accordingly, the bladders of all injured animals were expressed manually three times daily for the 1st week post surgery, then twice daily and gradually discontinued as the voiding reflex progressively returned by the end of the second week. The skin is constantly monitored to avoid development of pressure sores, a complication associated with immobility and the lack of ability of the skin to detect noxious sensations due to nerve impairment. The skin was inspected three times daily for the first week and twice daily subsequently until the surgical wound was completely healed.

Unlike in humans where pressure sores are the predominant skin lesions, this is rarely the case in animals since even completely paraplegic animals display notable mobility in their cages. On the other hand, self inflicted lesions in the skin are more common and maybe attributable to impaired perception of body parts due to lack of sensation or non intentional excessive grooming of the body parts for the same reason. The lesions are usually treated by applying antibiotic

ointments or powder. In rare cases the animal may require surgical repair of the skin defect or it may be euthanized due to extreme self-afflicted injury.

4.5 Experimental treatment groups and drug administration

Treatment onset was delayed by two weeks from the injury time for all experiments. As mentioned earlier (section 2.3.3), the decision to use the 2 weeks delay period was based on previous pilot study findings in the lab. Our lab has already published findings from earlier treatment start points (as early as 1 hr post surgery) (Schulte et al., 2003).

Experimental Treatment groups	Sub-groups	Surgery performed	Treatment start time	Treatment schedule
3 days (3 dy)	Sham (n=6)	Laminectomy	None	None
	Quercetin treated (n=6)	SCI	2 weeks post surgery	75µmol/kg td i.p for 3 days
	Saline control (n=6)			Saline td i.p for 3 days
1 week (1 wk)	Sham (n=6)	Laminectomy	None	None
	Quercetin treated (n=6)	SCI	2 weeks post surgery	75µmol/kg td i.p for 1 week
	Saline control (n=6)			Saline td i.p for 1 week
2 weeks (2 wk)	Sham (n=6)	Laminectomy	None	None
	Quercetin treated (n=6)	SCI	2 weeks post surgery	75µmol/kg td i.p for 2 weeks
	Saline control (n=6)			Saline td i.p for 2 weeks
Naïve n=6	Healthy animals			

Table 4.1: Summary of various experimental groups and treatment protocols used to establish the therapeutic effect of delayed quercetin treatment in spinal injury.

In this study the spinal cord injured and sham animals were placed into three major experimental groups, based on the treatment duration. The treatment regimen of 75 µmol of

quercetin per kg weight or saline vehicle only (control) was implemented to the spinal cord injured animals for a period of 3 day (3 dy), 1 week (1 wk) and 2 weeks (2 wk) starting at two weeks post surgery. The sham animals received no treatment and served as a control for the surgery. Therefore each major group consisted of three sub-groups (spinal injured quercetin treated, spinal injured saline controls and sham surgery controls). In addition, one group of healthy animals served as a naïve control for all animals and served mainly to validate intact locomotor function in the sham group. Table 4.1 provides a summary of the various experimental treatment protocols used in this study.

The drug was dissolved in 1 ml of saline solution (suspension) and was administered by intraperitoneal injection every 12 hr with 25 GA polypropylene hub hypodermic needle (Tyco Healthcare Group LP Mansfield, MA). All treatment started at two weeks post injury, i.e. on the first day of the third week after surgery.

4.6 Evaluation of locomotor function recovery

For all experiments, the animals were acclimatized for handling and test situation for five minutes twice daily for at least five days before the surgery. Usually by the third day we could notice good adaptation to the environment with less shyness and more willingness to explore the new spaces.

Behaviour testing was done by means of the modified inclined plane scoring system (Rivlin and Tator 1977) and the Basso-Beattie-Bresnahan (BBB) open field locomotor rating scale (Basso et al., 1995). Assessment was done by two individuals who were blinded to the experimental settings. Locomotor function was assessed on day one post surgery and then weekly for a minimum period of eight weeks after surgery.

4.6.1 The Inclined plane scale

For quantitative assessment of the rat's functional ability, we used the modified incline plane scoring system adapted from Rivlin and Tator (Rivlin and Tator 1977). This scoring system, which was first described by Eidelberg and colleagues (Eidelberg et al., 1976), used an inclined ramp to assess locomotor activity in ferrets. The system was subsequently adapted for rats by Rivlin and Tator in 1977. Unlike the original Eidelberg system, where the animals were first trained to run up the ramp, the modified scoring system does not require any training. Rats are placed horizontally on a movable inclined plane and the maximum inclination at which it can maintain itself for at least five seconds is recorded as the rat's functional ability. To maintain themselves on the plane, the animals use both forelimbs and hind limbs. These measurements were done on the first post-operative day and then weekly for up to eight weeks after surgery.

Rivlin and Tator reported an average score of about 80° in normal animals compared to an average of 23° in the injured animals. In our experience the healthy animals had an average response between 45° and 55°. It is important to take into account that the angle at which healthy (uninjured) rats can maintain position on the plane also depends on the surface material that is fixed to the board of the movable plane. Differences in scores in other laboratories are most likely due to differences in the surface of the incline plane board used.

A practical advantage of this inclined plane (IP) method is its rapidity and ease of use. It is inexpensive and offers a reliable quantitative measure of locomotor function. However, although the results of this testing system give us quantitative assessments of the strength of the hind limb, they do not provide significant information about complex somatic motor functions such as posture and voluntary limb positioning and coordination. To complement for these differences we also adopted the BBB scale described below.

4.6.2 The BBB locomotor rating scale

The BBB score categorizes combinations of rat hindlimb movements, trunk position and stability, stepping, coordination, paw placement, toe clearance and tail position, which all represent sequential recovery stages that rats attain after spinal cord injury (Basso et al., 1995; Basso et al., 1996). This scoring system has been evaluated by the Multicenter Animal Spinal Cord Injury Study (MASCIS) trial (Basso et al., 1996) and is currently used extensively throughout the neurotrauma literature (Kamencic et al., 2001; Schültke et al., 2003; Joshi and Fehlings 2002; Zhang et al., 2007; Wong et al 2009; McGirt et al., 2009; Schültke et al 2010).

The BBB scoring system is an operationally defined scale that uses a point system from 0 to 21 to assess locomotor functional recovery of the hind limbs after thoracic spinal cord injury in rats. This rating is made on an ordinal scale (i.e. the scale does not have equal intervals between the points) and has a very wide range, with 0 denoting no observable movement in the hind limbs, 7 representing extensive movement of all hindlimb joints, 15 corresponds to consistent plantar stepping and co-ordinated limb movement with no or occasional toe clearance, while 21 describes a perfectly healthy, walking animal. A detailed description of the BBB scoring system is provided in table 4.2 below.

Animals were pretrained to locomote in an open field consisting of a plastic pool of approximately 90 cm wide and 10 cm high walls. Two trained individuals blinded to the study then tested the animals for 5 min and rated them using the 21-point scale. All animals were tested in an identical manner prior to surgery and received a BBB score of 21. After surgery, the animals were tested at day 1 post-surgery and twice weekly up to the 8 week end point. The mean value for each animal per week was obtained by averaging the highest scores assigned by both individuals for each animal per week.

Score	Verbal description of hind limb movement
0	No observable hind limb (HL) movement
1	Slight movement of one or two joints
2	Extensive movement of one joint <i>or</i> extensive movement of one and slight movement of another joint
3	Extensive movement of two joints
4	Slight movements of all three joints of the hind limbs
5	Slight movements of two joints and extensive movement of the third joint
6	Extensive movements of two joints and slight movement of the third joint
7	Extensive movement of all three joints
8	Sweeping without weight support <i>or</i> plantar paw placement without weight support
9	Plantar paw placement with weight support in stance only or occasional, frequent or consistent weight supported dorsal stepping and no plantar stepping
10	Occasional weight supported plantar steps, no fore limb (FL) - hind limb (HL) coordination
11	Frequent to consistent weight supported plantar steps and no FL - HL coordination
12	Frequent to consistent weight supported plantar steps and occasional FL - HL coordination
13	Frequent to consistent weight supported plantar steps and frequent FL - HL coordination
14	Consistent weight supported plantar steps, consistent FL -HL coordination; predominant paw position during locomotion is rotated when it makes initial contact with surface or before lift up at the end of stance. Or frequent stepping, consistent FL -HL coordination and occasional dorsal stepping
15	Consistent plantar stepping and FL. HL coordination; no toe clearance during forward limb advancement; paw position predominantly parallel to body at initial contact
16	Consistent plantar stepping and FL. HL coordination; toe clearance occurring frequently during forward limb advancement; paw position predominantly parallel to body at contact and rotated at lift off
17	Consistent plantar stepping and FL. HL coordination; toe clearance frequently during forward limb advancement; paw position predominantly parallel to body at contact and lift off
18	Consistent plantar stepping and FL. HL coordination; toe clearance consistently during forward limb advancement; paw position predominantly parallel to body at contact and rotated at lift off
19	Consistent plantar stepping and FL. HL coordination; toe clearance consistently during forward limb advancement; paw position predominantly parallel to body at contact and lift off and tail down part or all of the time
20	Consistent plantar stepping and FL. HL coordination; consistent toe clearance; paw position predominantly parallel to body at initial contact and lift off; tail consistently up; trunk instability
21	Healthy animal - consistent plantar stepping, and gait coordination, consistent toe clearance, predominant paw position is parallel throughout stance, tail up, trunk stability

Table 4.2: The 21 -point Basso Baettie Breanahan (BBB) locomotor rating scale (Basso et al, 1995)

Some advantages of the BBB rating system include the simplicity of the testing apparatus and the use of clearly defined criteria to assess locomotor behavior. However, statistical analysis of the quantitative data has presented researchers with some complexity. There has been little agreement as to the statistical method to be employed to assess group differences. Both non-parametric and parametric statistic tests have been employed with the BBB scoring system (Joshi et al 2002, Lankhorst et al, 1999, Scheff et al., 2002, Basso et al 2002).

4.6.3 Statistical analysis

Non-parametric tests are usually recommended for use with ordinal data. However, in the case of behavioral data such as the BBB scale the use of parametric statistics has been recommended (Scheff et al, 2002). The use of non-parametric statistics with ordinal data is based largely on the properties of the distribution that occur with ordinal data. When ordinal data points are ranked from highest to lowest the resultant distribution of scores assumes a rectangular shape. This is because each possible score occurs exactly once and therefore a histogram displaying the data shows a set of frequency bars all at the same height. Hence, the assumption that the distribution of data points is approximately normal (which governs parametric statistics) is violated. However, in the case of locomotor behavior, the data are not truly ordinal as more than one subject may score the same rank during the behavioral observations. In this case, the data collected produces a distribution that is not rectangular but for the most part approximate normality. Hence, parametric statistics which rely on the assumption of normality can be used for behavioral data.

A normal distribution is defined by two population parameters, which are the mean and standard deviation. Parametric tests are based on these parameters. The most common parametric test used to compare multiple group differences is the analysis of variance (ANOVA). ANOVA

test is governed by two assumptions which include the assumption of homogeneity and sphericity of variance. It should also be pointed out that ANOVA is a very robust test capable of withstanding violations of its assumptions (Scheff et al., 2002; Howell, 2007). Violations of the assumption of homogeneity (i.e. variance of scores for each population is equal) are not critical as long as the largest variance is no more than 4 times larger than the smallest variance and the sample size is approximately equal. In the same way, the robustness of the ANOVA renders it capable of withstanding violations of the assumption of sphericity (variance of means in any level of the between-subject factors is equal to the variance of means in every other level of that factor).

In this study we used parametric statistics to determine significance in both BBB and incline plane scores, because the dependent measure did not present major violation to the assumptions on which ANOVA is based. Two-way-ANOVAs were done followed by Bonferoni post hoc test to determine if there were significant differences at each observed time point between saline and quercetin treatment within groups. Significant differences between treatment duration time points for the various quercetin treatment regimens at the 8 week endpoint were also determined using one-way ANOVA. A p value of <0.01 was considered to be statistically significant. Values were expressed as mean \pm standard error of means (SE).

4.7 Tissue collection and preparation

At the completion of treatment period and locomotor testing, all animals were deeply anaesthetized with 5% halothane in oxygen and perfused via the left ventricle with cold saline (Abbot Animal Health Saint Laurant Quebec). The spinal cords were harvested using the spinal roots as land marks (T4-T8) and all the samples collected from each animal (~9-10mm) included

the segment containing the site of the lesion and two segments rostral and caudal to the injury site. The segments harvested were used for Western blotting, histology, TUNEL and immunocytochemical analyses. A representative photograph showing the spinal cord tissue fragment harvested containing the injury site is shown in fig. 4.2.

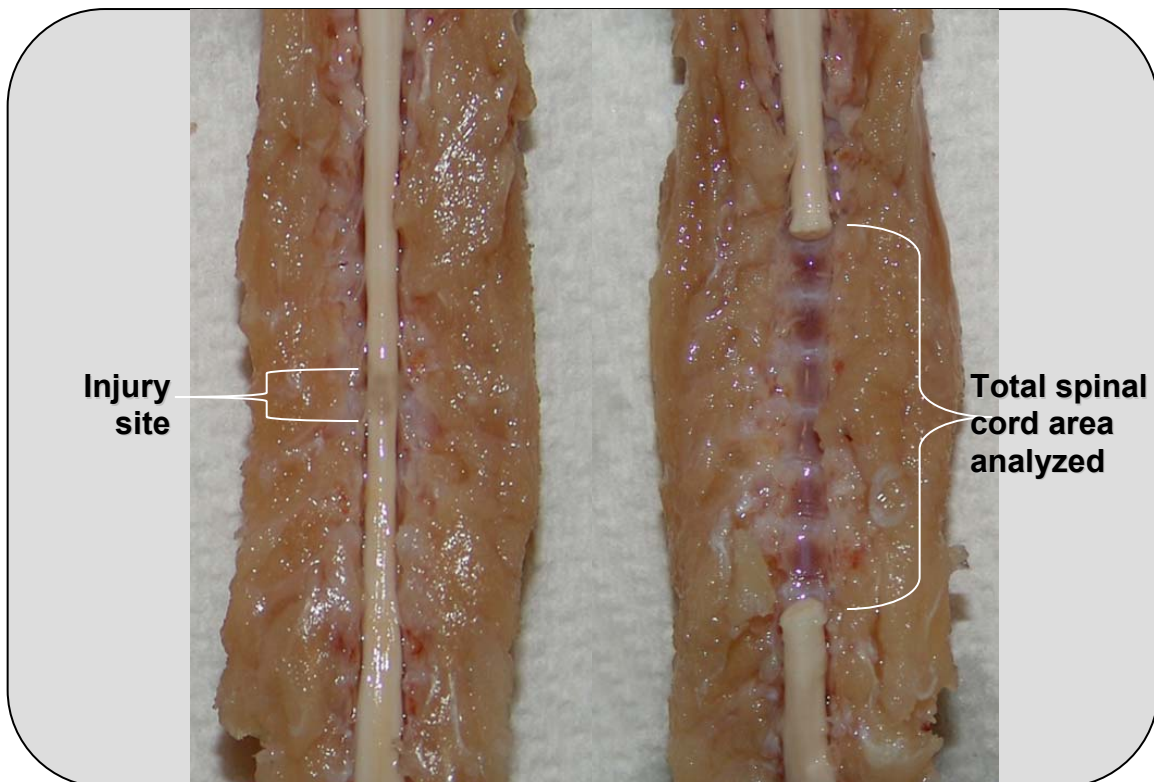


FIGURE 4.2: Representative spinal cord tissue fragment harvested at the injury site for various experimental analyses

4.7.1 Tissue preparation for Western blot analysis

To obtain samples for western blot analysis the spinal cords were harvested at the site of injury and carefully isolated in a cold medium (ice-cold PBS) then placed into labelled freezer resistant storage vials (VWR international Ltd, Canada) and snap frozen in liquid nitrogen. The frozen pieces were stored at -80°C until further processing. The tissues were later weighed and

crushed with mortar and pestle making sure to keep the tissue frozen in liquid nitrogen. Tissues were further ground with a Dismembrator (Mikro-Dismembrators B. Braun Biotech International) to a powder. The powder was then immediately transferred in labelled vials containing an iced-chilled solution made of an anti-protease cocktail (Sigma Aldrich, Oakville, Ontario, Canada) in PBS pH 7.4 (0.5-1 ml per 0.3 g of tissue). The samples were then stored at minus 80°C (- 80°C) until further processing. At a later date, samples were allowed to gradually thaw by placing them on a beaker of cold ice. Tissue was then sonicated using a pulse sonicator (Branson Ultrasonic Corporation, USA) three times for three seconds making sure to keep the sample cold in a beaker of iced water. Samples were centrifuged at 12000 g for five minutes at 4°C. A Bradford assay was conducted on the lysate to determine the protein concentration so that an equivalent quantity of protein could be loaded onto each gel. Next, a sample (loading) buffer solution (5x loading buffer: 0.5M Tris HCL pH 6.8, 10% SDS, 50% glycerol, 0.05% bromophenol blue and 20x reducing agent: 2M DTT) was prepared and the amount to be added to each sample was calculated based on the protein concentration. The loading mixture for each sample was then prepared by adding the sample and the corresponding volume of sample buffer. Samples were then denatured by heating at 95°C for 5 minutes and then centrifuged at 13,00 g for 30 seconds. The denatured protein samples were either promptly loaded on SDS-PAGE for electrophoretic separation and Western blotting or stored at -20°C to continue the next day.

4.7.2 Tissue preparation for Histology, TUNEL and Immunocytochemistry

4.7.2.1 Sample preparation: To obtain samples for histology and TUNEL staining and immunocytochemical studies, perfusion with saline was followed by 4% formaldehyde (prepared from paraformaldehyde) in phosphate buffered saline (PBS pH 7.4). The spinal cord was isolated at the injury site using spinal root landmarks and the samples were

post-fixed in 4% formaldehyde for 48 hrs at 4°C. Prior to embedding, all the spinal cord samples collected from each animal (~9-10mm) were cut into three parts which included a piece containing the site of the lesion and the pieces rostral and caudal to the injury site. All samples were labelled and embedded such that sectioning would begin on the side closest to the lesion.

4.7.2.2 Embedding and sectioning: Embedding of tissue samples was done either with paraffin or with the cryostat preserving gel O.C.T (optimal cutting temperature) (Tissue-Tek® O.C.T™ Compound, Miles, Elkhart, IN). The two methods of embedding have their advantages and disadvantages. Cryopreservation is good for preserving antigen epitopes, therefore, ideal for studies requiring antigen antibody reaction. Processing for paraffin embedding may disguise the epitopes but will provide better structural histological details. Hence, paraffin embedding was used prevalently for samples destined for histology and morphology studies while O.C.T compound was used for the most part for tissue destined for immunocytochemical and TUNEL analysis.

Spinal cord samples destined for cryostat sectioning were first cryoprotected by immersion in 30% sucrose at 4°C for 24 hrs. Each sample was then embedded with the embedding compound O.C.T. gel and rapidly frozen by placing in a container of dry ice (-70°C) to form frozen blocks for cryostat sectioning. Since spinal cords collected from each animal were cut into three pieces, we obtained three embedded blocks from each animal. Blocks were kept at -80°C until sectioned. Serial cross sections of 8 µm were cut at -20°C using a cryostat and mounted on Superfrost plus® (VWR international Ltd, Canada) slides. Mounted slides were numbered and stored at -20°C until immunolabelling.

The samples destined for paraffin embedding were first washed in PBS then immersed in 70% alcohol before being placed in a tissue processor (Tissue-Tek®VIP, Miles Scientific) to undergo serial dehydration prior to embedding into paraffin blocks. Similar to in cryopreservation, three embedded paraffin blocks were obtained for every animal. Serial sections of 5-10 µm (alternating four slides of 10 µm sections for every four slides of 5 µm sections) were taken transversally to the spinal cord axis and mounted on gelatine coated slides (VWR International Ltd, Canada). There were two sections per slide. Slides were stored in slide boxes at room temperature until stained with hematoxylin and eosin (H and E), luxol fast blue (LFB) or toluidine blue (TB). Some paraffin sections were also used for TUNEL labelling and Immunolabelling with DAB (section 4.11.2.1).

4.8 Western Blotting Analysis

4.8.1 Antibodies

Primary antibodies used for western blotting included anti-activated caspase-3, anti PARP (poly (ADP-ribose) polymerase), anti-GFAP (Glial fibrillary acidic protein), anti-ED1, anti β-AP (beta Amyloid peptide), anti OX-42, anti-β-Actin, a variety of phosphorylated MAPK including anti-phospho SAPK/JNK, ERK 1/2, p38, and their corresponding total MAPK (anti-MAPK SAPK/JNK, ERK 1/2 and p38). Anti-β-Actin was used as the internal standard for all antibodies except for the phosphorylated MAPK kinases where the total MAPK was probed as internal control. All antibodies were diluted in 50% blocking solution (2.5% skimmed milk powder).

Anti-Caspase-3 (Cell Signaling Technology, Inc; # 9664) a rabbit monoclonal antibody that recognizes only activated caspase-3 was used at a dilution of 1:1000 to assess the effect of quercetin administration on caspase-3 activation.

Anti-PARP (Cell signalling Technology, Inc; # 9542) a rabbit polyclonal antibody that specifically detects endogenous levels of full length PARP1 (116 kDa), as well as the large fragment (89 kDa) and small fragment (24 kDa) of PARP1 resulting from caspase cleavage was used at a 1:600 dilution to assess the effect of quercetin on PARP cleavage in vivo. Proteolytic cleavage of PARP by caspases is a hallmark of apoptosis.

Anti GFAP, (Abbiotec, San Diego California; # 250661) rabbit polyclonal antibody a cell-specific marker that distinguishes astrocytes from other glial cells was used at a dilution of 1:1000 to study the effect of quercetin on astrocyte activation.

Anti OX-42 (CD11) (mouse anti Cd11, Serotec, Inc.; # MCA275G) a mouse monoclonal antibody that recognizes activated microglia cells, was used at a dilution of 1:800 to determine the effect of quercetin on microglia activation.

Anti-EDI (mouse anti rat cd68, Serotec, Inc.; # MCA341R) a mouse monoclonal antibody that recognizes microglia activation was used at a dilution of 1:800.

Anti- β -APP, (beta-amyloid precursor protein) (Santa Cruz Biotechnology, Inc; # sc-9129) a mouse polyclonal antibody that recognizes beta amyloid aggregation and deposition in CNS was used at a dilution of 1:1000 to study the effect of quercetin on impairment of axoplasmic transport.

Anti-Phospho MAPK p38, Phospho MAPK p42/42 and Phospho SAPK/JNK (Cell Signaling technology, Inc; sampler kit # 9910), all rabbit monoclonal antibodies that recognize only activated (phosphorylated) forms of MAPK p38, ERK 1/2 and SAPK/JNK, were used at a

dilution of 1:500, 1:1000 and 1:1000 respectively to assess the effect of quercetin on MAPK activation *in vivo*.

Anti-MAPKs p38, ERK 1/2 (p42/42) and *SAPK/JUNK* rabbit monoclonal antibodies (Cell Signaling technology, Inc; sampler kit # 9926) that recognize endogenous levels of the total forms (i.e. phosphorylated and non phosphorylated) of MAPK p38, ERK 1/2 and SAPK/JNK were used at a 1:1000 dilution and probed as internal standard for the corresponding activated forms of MAPK kinases.

Anti- β -Actin mouse monoclonal antibody (Sigma-Aldrich, Inc. Canada; # C5838) that recognizes the cytoskeletal protein β -actin in a wide variety of tissues was used at a dilution of 1:5000 and probed as internal control for all antibodies except for MAPK kinases.

4.8.2 Western blotting technique

Spinal cord tissue was harvested and prepared for Western blotting as previously described (see section 4.7.1). Protein samples were separated by SDS-PAGE (Criterion XT gels and cell unit, Bio-Rad laboratories). Following electrophoresis, proteins were electroblotted onto nitrocellulose paper (Bio-Rad) using a wet transfer unit (Criterion Blotter, Bio-Rad laboratories) using the manufacturer's instructions. Equivalent loading was verified by Ponceau S dye staining after transfer of the SDS-PAGE. To prevent non-specific association of antibodies the blots were blocked with 5% skim milk powder (Bio-Rad laboratories) overnight at 4°C followed by incubation with the primary antibody in PBS containing 2.5% skim milk powder overnight at 4°C. Blots were then washed three times for 5 min with blocking solution (5% skimmed milk powder) followed by incubation with horseradish peroxidase (HRP) linked secondary antibody for 45 min at room temperature. Blots were again washed three times in 5% skimmed milk and then three times in PBS. A chemiluminescence kit was used to visualize the bound HRP-

conjugated secondary antibody (PerkinElmer Life Sciences, Boston, MA). The membrane was exposed to Kodak X-Omat AR film and developed.

4.8.3 Data collection and statistical analysis

The films developed from Western blot analysis were scanned using a high performance scanner (Umax Astra 2400S) connected to a Macintosh computer equipped with software (Adobe Photoshop) that converts captured images into tiff format. Blots were quantified using the NIH Image-J software. Bands were quantified as the intensity of the investigated band divided by intensity of its corresponding standard keeping the same area per signal. β - actin was used as standard for all investigated antibodies except for the MAPK antibodies where the phosphorylated bands were standardized to that of the total MAPK.

Quantitative data pertaining to protein analysis expression obtained for all treatment groups were analyzed with analysis of variance (ANOVA) with Bonferoni post hoc analysis to determine whether significant differences existed between quercetin and saline treatments. ANOVA also determined whether there were any significant differences between the quercetin treated in various treatment regimens. Values were expressed as mean \pm standard error (SE). A p value of <0.01 was considered statistically significant

4.9 Histology

Tissues were prepared and sectioned as previously described (see section 4.7.2). Every other third, fourth and fifth slide from paraffin embedded tissues was stained respectively with hematoxylin and eosin (H and E), luxol fast blue (LFB) and toluidine blue (TB).

4.9.1 Hematoxylin and Eosin (H and E) staining

The H and E staining was adapted from Humason's Animal tissue techniques, (1979) (see appendix 5). This procedure is straightforward and exploits the basic and acidic affinities of its component dyes to stain tissue structures. The hematoxylin dye which is a base interacts with acidic structures (such as nucleic acid) staining them in shades of blue while the acidic dye eosin, interacts with basic tissue to stain them in shades of pink. This stain gives a good picture of histological orientation of tissue structures and permits a good visualization of cavities formed in injured spinal cords. However, it is limited by the fact that it gives only minimal information about the chemical makeup of a tissue or organ. We used H and E staining to observe and evaluate cavity formation, structure and tissue orientation in spinal cord injured rats following experimental treatment with quercetin or saline vehicle only.

4.9.2 Toluidine blue (TB) staining

The TB staining procedure was adapted from Raphael's Lynch's Medical laboratory technology (1983) (see appendix 6). Toluidine blue is basic blue dye. Similar to H and E this stain provides good details of orientation of tissue structures under light microscope. It also provides very little information about the chemical structure of the tissue. TB is often used clinically as a specific stain for mast cell tumours because of its strong affinity the glycosaminoglycans in the mast cell granules. In the presence of glycosaminoglycans the TB undergoes a metachromatic shift in colour. We used the TB stain as a complimentary staining to H and E to assess cavity formation, spinal cord tissue preservation and structural organization in quercetin treated and saline control spinal cord injured rats.

4.9.3 Luxol fast blue staining (LFB)/Cresyl violet (Nissl stain)

This stain first described by Kluver and Barrera (1953), (taken from our department's "Laboratory notes in Histology Techniques" –Anatomy 412B 1982; see appendix 7) is designed to show myelin (LFB) and Nissl substance (cresyl violet). LFB stains the myelin sheath blue while cresyl violet stains the Nissl substance pink to violet appearance. LFB stained sections were used to assess spinal cord tissue myelin organization and white matter sparing in quercetin treated and saline control spinal cord injured animals. In addition, sections from LFB stained slides containing the epicentre of the injury were used for white matter quantification.

4.9.4 Image analysis

Images of histology stained slides (H and E, LFB and TB) were captured using a light microscope (Carl Ziess Leica LEITZ DMRD) coupled with a Sony® DSV-V3 digital camera that converts images into tiff and jpeg formats. Images from digital camera were then transferred to a Macintosh computer as TIFF format.

4.9.5 White matter quantification

Slides to be used for white matter quantification for all animals were selected LFB slides (10 µm sections) taken from the middle piece of paraffin embedded spinal cord (~3 mm) which contained the injury area for each animal (n=6 per treatment regimen). In this way we could narrow down to slides containing the epicentre of the injury. Luxol fast blue images to be quantified were all captured at 5x magnification to include the entire spinal cord cross section. Semiquantitative assessment of LFB stained slides was done by visual assessment of slides to ensure that the sections contained the epicentre of the injury and six slides per animal were selected and analyzed for amount of LFB positive tissue. Quantification was done using the NIH Image-J software with a color deconvolution plug-in (Ruifrok and Johnston, 2001). The color

deconvolution plug-in was run using the Fast Blue, Fast Red and DAB program, subtracting the background to bring the image to threshold. The quantification tool was then used to calculate the number of stained pixels. The analysis was run in triplicate and the average of the three measurements was used as the value for the sample. The average number of pixels for the total cross sectional area of the thoracic sections from six healthy (uninjured) animals was measured. Calculations of white matter sparing for the injured animals were performed by dividing the number of LFB positive pixels over the number of pixels in the total cross sectional area of the healthy (uninjured) controls. Results were displayed as average percentage \pm standard error. Data were analyzed statistically using ANOVA with Bonferoni post hoc. A P value of <0.05 was considered statistically significant

4.10 TUNEL Method (*terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate (dUTP) nick end-labeling*)

This method is used to detect and quantify apoptosis (programmed cell death) at a single cell level based on DNA strand breaks. The fragmentation of genomic DNA is a typical biochemical hallmark of apoptosis which is an irreversible event that commits the cell to die. In many systems, DNA fragmentation has been shown to result from the activation of endogenous Ca^{2+} and Mg^{2+} -dependent nuclei endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal unit (linker DNA) generating double stranded as well as single strand fragments (also called “nicks”) that can be identified by labelling free 3'-OH terminal with modified nucleotides in an enzymatic reaction. In the TUNEL method, labeled terminal deoxyuridine triphosphate (TdT) is enzymatically added to free 3'-OH group ends of DNA exposed during the cleavage process.

4.10.1 TUNEL Labelling Technique

Tissues were prepared and sectioned as previously described (see section 4.7.2). The experiments were run on tissue harvested at the 8 week study endpoint from two treatment regimens (3 day and 2 week treatment regimen). TUNEL labelling analyses were done on both cryostat sectioned and paraffin sectioned tissue. Experiments were run in triplicate (twice with cryostat sections and once with paraffin sections).

Slides from paraffin sectioned tissue were incubated in DAB for light microscopy viewing whereas the cryostat sectioned tissues were fluorescent-labelled. Slides with paraffin embedded sections were dewaxed and rehydrated according to standard protocols. Frozen slides from the freezer were first kept to equilibrate to room temperature for 1 hr before beginning the analysis.

Next, slides were immersed in freshly prepared 4% formaldehyde in phosphate buffered saline (PBS) for 30min at room temperature on a shaker followed by three cycles of 10 min wash with phosphate buffered saline plus 0.05% Tween 20 (PBST). Slides were then incubated for 20 min in 10 ug/ml proteinase K in PBS (at room temperature) followed by three washes in PBST for 5 min. Slides were subsequently post-fixed for 30 min with 4% formaldehyde in PBS and again washed three time for 5 min in PBS. Next, slides were permeabilized in a solution containing 0.1% Triton-X in 0.1% sodium citrate in 0.01M PBS at 4°C for 10 min followed by another three washes in PBS for 5 min.

At this point, an additional step was required only for slides intended for DAB incubation (slides were immersed in a solution of 3% hydrogen peroxide (H₂O₂) in methanol for 30 min to block the endogenous peroxidase followed by three cycles of 5 min washes in PBST) otherwise the slides were ready for washing and incubation in TUNEL buffer. All slides were thereafter

washed for 5 minutes in TUNEL buffer (100 mM Cacodylic acid, 2.5 mM cobalt chloride, 0.1 mM dithiothreitol, 0.1 mg/ml of BSA, 0.01% Triton X-100) then incubated in a change of the same buffer for 1 hr with gentle shaking at 4°C. The TUNEL reaction mixture consisting of the TUNEL enzyme + TUNEL label (nucleotide labelling mixture for the TUNEL reaction) was prepared according to the manufacturer's instructions (Roche Diagnostics®; # 1 684 795) and added to tissue on slides followed by incubation in a dark humidified chamber for 3 hrs at 37°C.

Two positive controls and negative control slides were included in each experimental setup and were prepared as follows; *positive controls*: sections were incubated with DNase I, grade I (3000 U/ml in 50 mM Tris-HCl at pH 7.5 + 1 mg/ml BSA) for 10 min at 15-25°C to induce DNA strand breaks prior to labelling. *Negative controls*: sections were incubated for 1hr at 37°C with TUNEL label solution only (without enzyme terminal transferase) instead of TUNEL reaction mixture. All slides were then washed three times for 5 min in PBS then allowed to air dry.

4.10.1.1 Labelling for fluorescence microscopy viewing: Slides destined for fluorescent viewing were then incubated in Hoechst stain (0.5µg/ml) (Sigma Aldrich Inc Canada; # H6024) for 10 min at room temperature. Hoechst stain intercalates with DNA allowing visualization of nuclei. This was followed by two 5 min washes in PBS then air dried and cover-slipped with Citiflour mounting medium (Marivac Ltd, Halifax, NS, Canada) followed by viewing under the fluorescence microscope and photography. Slides were stored in dark to at 4°C prevent decay of fluorescent signal.

4.10.1.2 Labelling for light microscopy viewing: To obtain a specific color reaction visible under the light microscope, fluorescent stained slides were immersed in TUNEL POD (anti-fluorescein-antibody) (Roche Diagnostics® # 1 684 817). TUNEL POD is a

secondary detection system that converts the fluorescent label into colorimetric signal and uses 3, 3'-Diaminobenzidine (DAB) as precipitating substrate. Slides were incubated in TUNEL POD (50 µl per slide) in a humidified chamber for 1 hr at 37°C followed by three washes for five minute in PBST. The DAB solution was prepared from the DAB kit (Vector Laboratories, CA, USA) according to the manufacturers instructions (by adding in an orderly manner 5 ml of distilled water, 2 drops of buffer stock pH 7.5, 4 drops of DAB substrate, and 2 drops of hydrogen peroxide). Next, slides were incubated in DAB solution (50-100 µl to each slide) for 5-10 min at RT (depending on desired intensity, usually at 6-8 min) and again washed three times for 5 min in PBS. Slides were then air dried, cover-slipped with Cytoseal mounting medium (Marivac Ltd, Halifax, NS, Canada) followed by visualization under light microscopy and photography.

4.10.2 Image analysis and data collection:

Images of DAB stained slides were captured using a light microscope and then transferred to a Macintosh computer as previously described (see section 4.9.4).

Fluorescent stained slides were visualized and captured using a high performance fluorescence microscope (Olympus IX 70) equipped with interchangeable filters for red, blue and green images viewing and connected to a computer (Compaq Diskpro) that uses software (SPOT Basic and Image Pro-Plus) capable of converting captured images into a tiff or jpeg format. TUNEL Images destined for quantification were captured at 20x then 40x magnifications without moving the slide. The 40x images were used for quantification analyses. Moreover, matching images for both TUNEL and Hoechst stain from the same section were captured by switching the fluorescent filter for each stain without moving the slide. The captured images were then saved in tiff format.

4.10.3 TUNEL quantification

TUNEL quantification was done by manual count of fluorescent images captured at the 40x magnification. To facilitate this task of counting, Adobe Photoshop software was used to further magnify the captured images. Images for TUNEL positive cell counts were captured from fluorescent stained 8 μm tissue sections. A total of 9 slides per animal were selected such that they were spaced approximately 1mm apart (n=6 per treatment regimen). Slides were visually assessed to pick out stained sections with the strongest positive signals. Once a section was chosen, three areas were selected in a pre-specified manner (Fig. 4.3) for capturing the image used for cell counting; this included a posterior, lateral and anterior portion of the cross section.

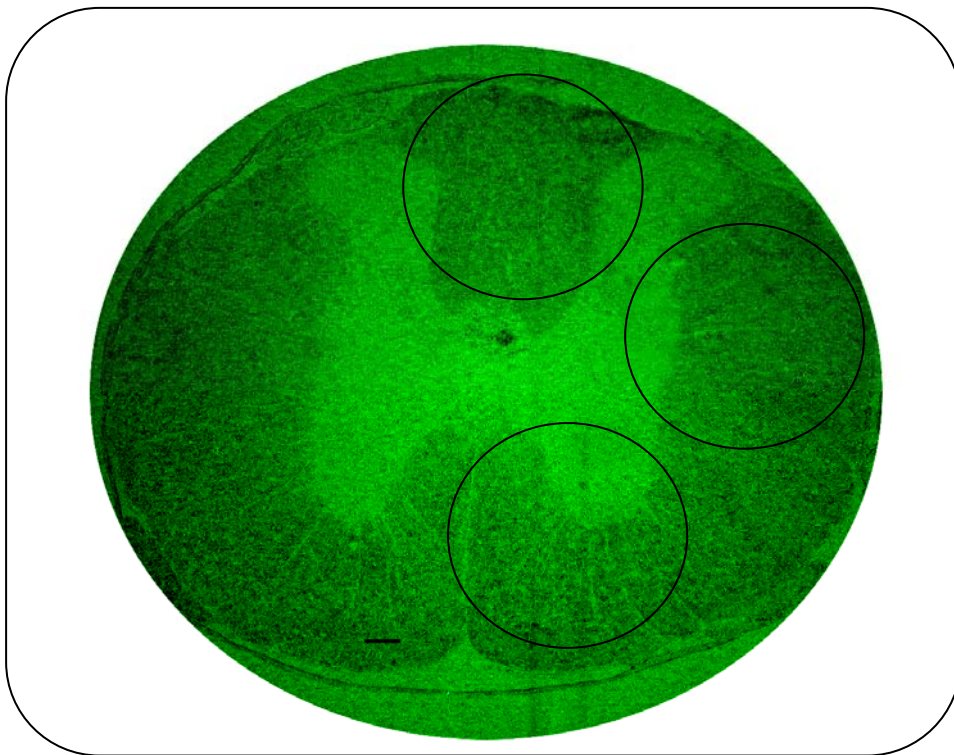


FIGURE 4.3: Schematic representation of areas analysed for TUNEL quantification in a positive control spinal cord section. Scale = 100 μm

The total TUNEL positive signals were expressed over the total Hoechst positive signals for each section counted and gave the value of the sample. The mean value of all nine samples gave the mean value for each animal. Values were expressed as mean ratio \pm SE. Values were analyzed with ANOVA and Bonferoni post hoc.

4.11 Immunocytochemistry

4.11.1 Antibodies and nuclear labelling agent

The same antibodies used for western blotting analysis (section 4.8.1) were analysed by immunocytochemical staining. These included antibodies against *activated caspase-3* (rabbit polyclonal anti caspase-3, Cell Signaling Technology Inc, 1:200; # 9664); *anti Cleaved PARP* (rabbit monoclonal anti cleaved PARP, Cell Signaling Inc, 1:200; # 9548); *anti-GFAP* (rabbit polyclonal anti GFAP Abbiotec, San Diego California, 1:300; # 25661); *anti ED1* (anti rat cd68, Serotec, Inc, 1:300; # MCA341R), *anti β -APP* (anti β -APP, Santa Cruz Biotechnology, Inc, 1:200; # sc9129), *anti OX-42* (mouse anti Cd11, Serotec, Canada, 1:200; # MCA275G) various *anti phosphorylated MAPK* (anti-phospho MAPK p38, phospho ERK 1/2 and phospho SAPK/JNK, Cell Signaling Technology Inc, 1:200; sampler kit # 9910) and *anti total MAPK* (anti-Total MAPK p38, ERK 1/2 and SAPK/JNK, Cell Signaling Technology Inc, 1:200; sampler kit # 9926). All were diluted in 50% blocking solution (2.5% skimmed milk powder). Hoechst stain (0.5 μ g/ml) (Sigma Aldrich Inc Canada; # H6024) was added to all slides to permit fluorescent viewing of cell nuclei.

4.11.2 Immunocytochemical technique

Tissues were prepared and sectioned as previously described (see section 4.7.2). A total of 9 slides per animal spaced approximately 1 mm apart were used for each

immunocytochemical experiment. Some experiments were run using frozen sections and were immunostained for fluorescent viewing while the other experiments used paraffin sectioned slides that were immunolabelled and incubated with DAB for light microscopy viewing. Slides with paraffin embedded sections were dewaxed and rehydrated according to standard protocols. Frozen slides from the freezer were first kept to equilibrate to room temperature for 1 hr before the experiment began.

4.11.2.1 Immunolabelling with DAB for light microscopy viewing

Primary antibodies used included, antibodies against phospholyted MAPKs (phospho MAPKp38, phospho ERK 1/2 and phospho SAPK/JNK), and activated caspase-3. Slides were dewaxed and rehydrated according to standard protocols. Next, slides were washed with PBS for 5 min and then immersed in a freshly prepared solution of 4 % formaldehyde in 0.3M PBS overnight at 4 °C on a shaker and then washed three times for 10 min in PBS. Slides were then immersed in a solution of 3% hydrogen peroxide (H₂O₂) in methanol for 30 min to block the endogenous peroxidase. This was followed by three cycles of 5 min washes in PBS. Next, slides were incubated in blocking solution containing 1% bovine serum albumin (BSA), 10% horse serum (HS), 1% Triton X-100 in 0.03 M PBS overnight at 4°C after which the solution was shaken off and sections were incubated with appropriate dilution in the primary antibody in 50% diluted blocking solution overnight at 4°C. This was followed by another 3 cycles of 5 min washes in PBS and then incubated with appropriate dilution of biotinylated secondary antibody in PBS for one hour at room temperature. Slides were again washed three times for 5 min with PBS. Next, slides were incubated with ABC reagent (Vector Labs) for 30 min at RT (2 drops each of reagents A and B in 10 ml of PBS) followed by three washes for 5 min in

PBS. DAB solution was then prepared using the DAB kit (Vector Labs) according to the manufacturers instructions (by adding in an orderly manner 5 ml of distilled water, 2 drops of stock buffer pH 7.5, 4 drops of DAB substrate, and 2 drops of hydrogen peroxide). After that, slides were incubated in DAB solution (50-100 μ l to each slide) for 5-10 min at RT (depending on desired intensity) and again washed three times for 5 min in PBS then rinsed off with running tap water for 10 min. Slides were then air dried and cleared for mounting with Cytoseal mounting medium (Marivac Ltd, Halifax, NS, Canada) and then left for 20 min to harden before being examined under the light microscope and photographed.

4.11.2.2 Fluorescent-Immunolabelling for fluorescence microscopy viewing

Primary antibodies used for this procedure included, antibodies against phosphorylated MAPKs (phosphoMAPKp38, phospho ERK 1/2 and phospho SAPK/JNK), activated caspase-3, PARP (Cell signalling Technology, Inc), GFAP, ED1 and β APP.

Frozen slides from the freezer were first kept to equilibrate at room temperature for 1 hr. Next, slides were washed with PBS for 5 min and then immersed in a freshly prepared solution of 4% formaldehyde in 0.3M PBS overnight at 4°C on a shaker and then washed three times for 10 min in PBS. Slides were then incubated in blocking solution containing 1% bovine serum albumin (BSA), 10% horse serum (HS), 1% Triton X-100 in 0.03 M PBS overnight at 4°C after which the solution was shaken off and sections were incubated with appropriate dilution in the primary antibody in 50% diluted blocking solution overnight at 4 °C. This was followed by another 3 cycles of 5 min washes in PBS and then incubated with appropriate dilution of biotinylated secondary

antibody in PBS for one hour at room temperature. Slides were again washed three times for 5 min with PBS. To allow for fluorescent visualization of cell nuclei slides were incubated in Hoechst stain (1 µg/ml) for 10 min at room temperature. This was followed by two 5 min washes in PBS then air dried and cleared for mounting with Citiflour (Marivac Ltd, Halifax, NS. Canada). Slides were then ready to be examined under a fluorescence microscope and photographed. Slides were stored in the dark to prevent decay of fluorescent signal.

4.11.3 Image analysis and data collection:

Since the same antibodies used for immunocytochemistry were investigated and quantified following Western blotting analysis, data from immunohistochemistry was limited to visual assessment without further quantification. The image data collected were used to complement the Western blot data. Images of DAB stained slides were captured using a light microscope as previously described (see section 4.9.4). Likewise, fluorescent stained slides were visualized and captured using a high performance fluorescence microscope equipped with interchangeable filters as previously described (see section 4.10.2). Matching images for the Hoechst stained epitope-antibody reaction could be captured from the same section by switching the filter for each stain without moving the slide. Slides were visually scrutinized to look for positive signals and pictures were taken and visually assessed for differences in reaction intensity between treatment groups.

Improved locomotor functional recovery following delayed treatment with quercetin in spinal cord injured rats is associated with decreased cell damage and neural tissue sparing.

5.1 Effect of quercetin on locomotor functional recovery following SCI

5.1.1 Introduction

Following surgery, all spinal cord injured animals acquired paraplegia and loss of bladder function while the sham operated animals showed no neurological deficits thus indicating that any observed deficits were attributable to the force of clip compression on the spinal cord. All sham animals scored the maximum BBB score of 21 and incline plane score of 50° within 24 hrs after surgery. Subsequent to the implementation of the treatment regimen two weeks after injury, we observed a significant improvement of locomotor function in the quercetin treated animals compared to the saline controls although severe deficits still remained in the quercetin treated. The three treatment regimens included a period of 3 days, 1 week and two weeks consisting of quercetin treated (n=6 per treatment regimen) and saline control (n=6 per treatment regimen). A sham group (n=6), which did not receive any treatment, was also added to each treatment regimen.

5.1.2 BBB scores

A plot of the average BBB scores following 3 days, 1 week and 2 weeks treatment regimens are shown below in figure (5.1 (a), (b), (c)). Since all sham animals attained the highest BBB scores achievable (21 points), their scores are only shown at the first observed time point

and omitted at later time points in each graph in order to display the results more clearly for the reader and highlight the differences between treatments (i.e. quercetin and saline). Likewise, sham animals were not included in the statistical analysis since the purpose was to show the differences between treatments. Increases in BBB scores could be observed already at one week after treatment initiation and continued to improve before reaching a plateau around the third week after treatment (5 weeks post surgery). This trend was seen in all three treatment regimens (i.e. treatment duration time points of 3 day, 1 week and 2 weeks). Comparison of the postoperative performances of the quercetin treated and saline control animals by analysis of variance (2-way-ANOVA with Bonferoni's post hoc) at each observed time point gave significant differences in all three treatment regimens by the first week after treatment ($p < 0.01$). This significant difference between treatments was maintained until the 8 weeks end point of the study.

At the end of the eight weeks study period, the highest BBB scores attained (refer to table 4.2 for description of neurological presentation associated with a given BBB score) within each treatment schedule were as follows (shown in table 5.1):

1. Within the 3 days treatment regimen, one of the quercetin treated animal ($n=6$) scored a high of 11, one scored 10 and four scored 9 points, while in the saline control animals ($n=6$) one scored a high of 1 point, two scored a high of 3 points and three animals scored a high of 2 points.
2. Within the 1 week treatment schedule, four from the quercetin treated group ($n=6$) scored highest of 10 points and two scored 9 points while in the saline control group ($n=6$) two animals scored 3 points, three animals scored 2 points and 1 animal scored 1 point.

3. Lastly, within the two weeks treatment schedule, two animals in the quercetin group (n=6) reached 10 points, three reached 9 points and one animal reached 8 points while in the saline control group (n=6), three animals scored 5, 3 and 2 points, respectively, while the remaining three rats scored 1 BBB point each.

	3 days Treatment regimen				3 days Treatment regimen				3 days Treatment regimen			
	Saline		Quercetin		Saline		Quercetin		Saline		Quercetin	
	2 wks	8 wks	2 wks	8 wks	2 wks	8 wks	2 wks	8 wks	2 wks	8 wks	2 wks	8 wks
BBB Scores	1	1	2	9	1	1	2	9	1	1	1	9
	2	3	1	10	2	3	1	10	2	5	1	10
	2	3	2	9	2	3	2	9	2	3	1	8
	1	2	2	9	1	2	2	10	2	1	2	9
	3	2	1	9	3	2	2	10	1	1	2	9
	2	2	2	10	2	2	2	10	2	2	3	10
Mean	1.833	2.16	1.666	9.333	1.833	2.16	1.833	9.666	1.666	2.166	1.666	9.16
std dev	0.752	0.75	0.516	0.516	0.752	0.75	0.408	0.516	0.516	1.602	0.816	0.75
std error	0.307	0.31	0.210	0.210	0.307	0.31	0.166	0.210	0.210	0.654	0.333	0.31

Table 5.1: BBB scores in saline control and quercetin treated animals in all treatment regimens before treatment (at 2 wks post surgery) and at the study end point (8 wks post surgery).

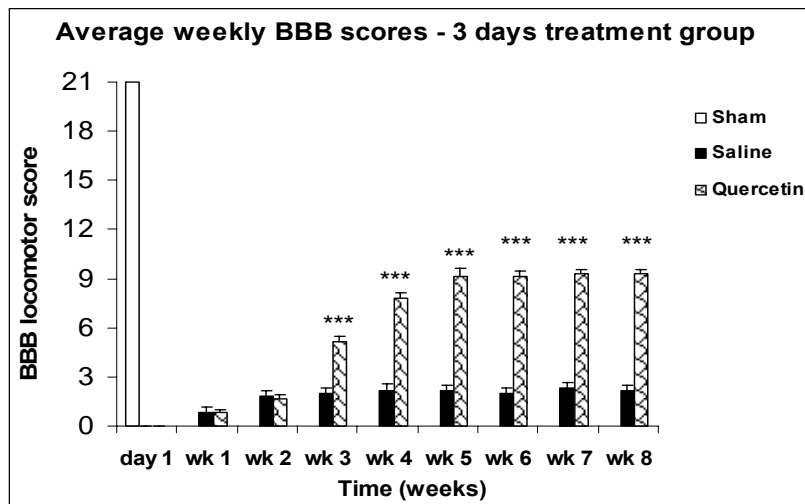


Fig. 5.1 (a)

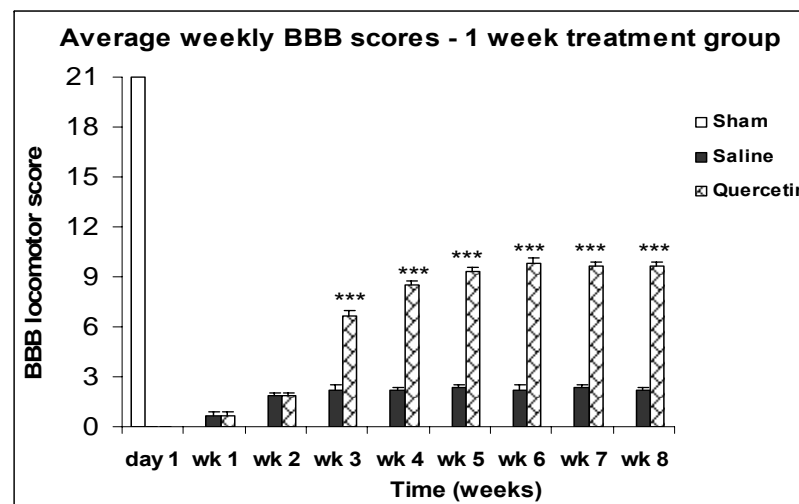


Fig. 5.1(b)

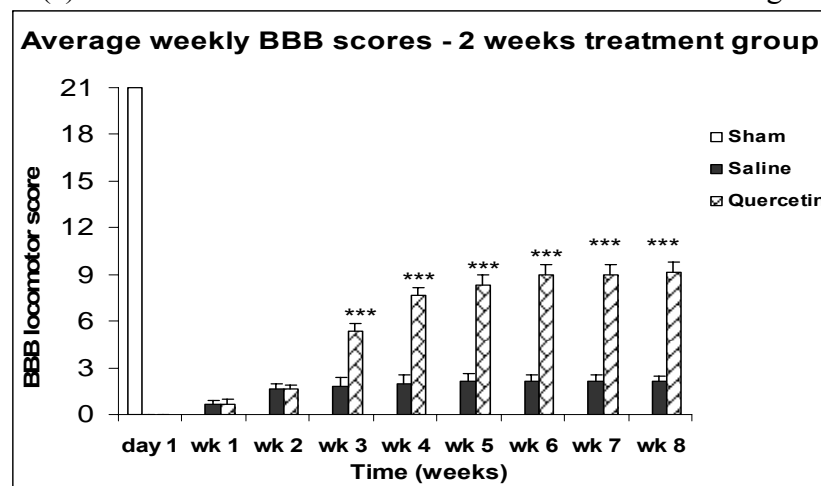


Fig. 5.1 (c)

FIGURE 5.1: Average weekly BBB open field locomotor scores following 3 days (a), 1 week (b) and 2 weeks (c) treatment regimen. Sham animals all show the highest score of 21. Significant increases were observed in quercetin treated ($n=6$) compared to saline control ($n=6$) as early as one week following treatment (3 weeks post surgery) and maintained throughout the study period. Values are expressed as mean \pm standard error (SE). ** and ***denotes statistical significance at $p<0.001$ and 0.0001 . $n=6$ animals per group for each treatment regimen.

Consequently, all animals in the quercetin treatment regime (i.e. 3 days, 1 week and 2 weeks treatment schedule) attained a BBB score of at least 8 “sweeping without weight support *or* plantar paw placement without weight support” with the highest value attained being a BBB score of 11 “frequent to consistent weight supported plantar steps and no FL - HL coordination”. None of the saline control animals reached beyond a BBB score of 5 points. A plot of the average BBB scores at the end of the study is shown in figure 5.2.

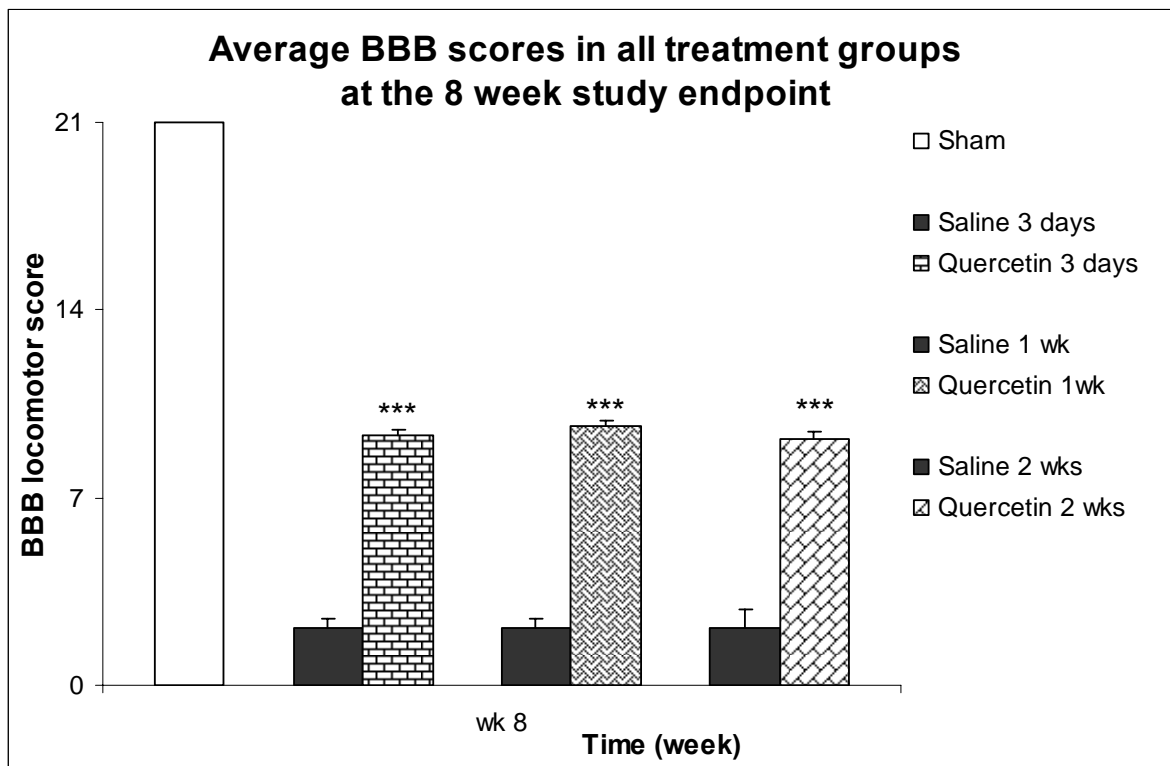


FIGURE 5.2: Average BBB score in all experimental groups at the 8 weeks study end point following various treatment regimens. Sham animals all maintained the maximum score. Remarkable improvement is observed in quercetin treated compared to saline controls. Values are expressed as mean \pm standard error (SE). ***denotes statistical significance at $p < 0.0001$. $n=6$ animals per group for each treatment regimen

Taken as a whole, slightly higher average BBB scores were observed in the quercetin treated animals that were on the 1 week treatment regimen compared to the 3 days and the 2 weeks duration time points. However, comparison of the treatment regimens using two-way-

ANOVA and Bonferoni's post hoc showed no statistically significant difference between the different treatment duration time points (Fig. 5.3) indicating that the duration of treatment had no statistically significant effect on the treatment outcome.

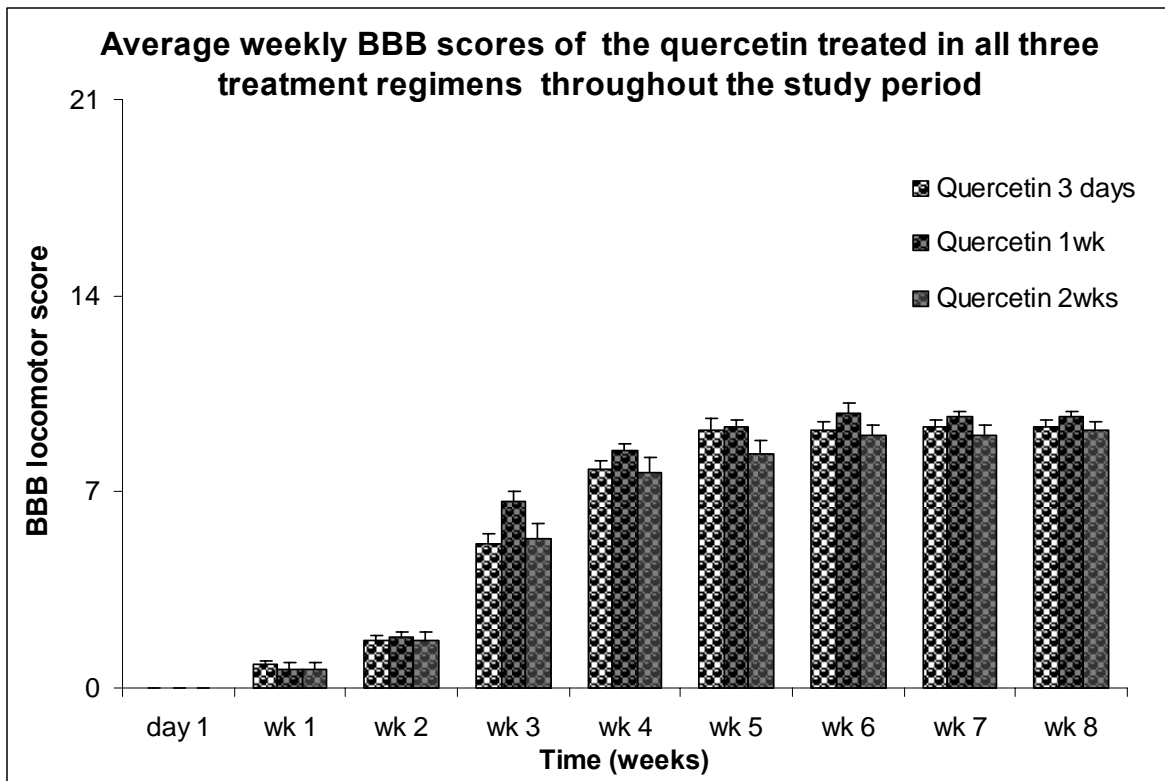


FIGURE 5.3: BBB scores in quercetin treated (n=6) animals in the three treatment regimens throughout the eight weeks study period. Higher BBB scores are observed in the 1 week regimen compared to 3 days and 2 weeks treatment regimens but ANOVA showed no statistically significant difference; indicating that the duration of the treatment had no statistically significant effect on the outcome of this study. Values are expressed as mean \pm standard error (SE). n=6 animals per group for each treatment regimen.

5.1.3 Incline plane scores

Similar to BBB scores, increases in incline plane values in the quercetin treated animals were also observed by the first week post treatment (3 weeks post surgery) and reached a plateau by the third week following treatment (Fig 5.4 (a), (b), (c)). Comparison of the post-operative performances by two-way ANOVA of the plane scores showed a statistically significant difference between the quercetin treated and saline control animals. Also, as with the BBB

scores, the results show no statistically significant difference amongst the various quercetin treatment regimens (i.e. 3 days, 1 week and 2 weeks) again indicating that the duration of treatment had no statistically significant effect on the treatment outcome.

The highest incline plane scores within each treatment regimen at the eight week study endpoint were as follows (shown in table 5.2):

1. Within the 3 days regimen, in the quercetin treated (n=6) the highest scores were respectively 35° (4 animals), 40° (2 animals) while in the saline control group (n=6), the scores were 20° (3 animals), 25° (2 animals) and 0° (animal).
2. Within the 1 week treatment regimen, the highest scores attained by the quercetin treated (n=6) were respectively 40° (3 animals), and 35° (3 animals), while in the saline control group (n=6) the scores were 20° (4 animals), 25° (1 animal) and 0° (1 animal).
3. Lastly, within the 2 weeks treatment regimen, highest scores in the quercetin treated were 35° (5 animals), and 40° (1 animal) while the saline control group (n=6) scored 0° (1 animal), 20° (4 animals), 25° (1 animal) respectively.

	3 days Treatment regimen				3 days Treatment regimen				3 days Treatment regimen			
	Saline		Quercetin		Saline		Quercetin		Saline		Quercetin	
	2 wks	8 wks	2 wks	8 wks	2 wks	8 wks	2 wks	8 wks	2 wks	8 wks	2 wks	8 wks
Incline Plane Scores	0	0	20	35	0	20	20	40	0	0	0	35
	15	25	15	40	20	20	20	40	20	25	0	35
	15	20	20	35	15	0	20	35	15	20	20	35
	0	20	20	35	15	20	25	35	20	20	15	35
	20	25	0	35	25	25	0	35	0	20	20	35
	20	20	20	40	0	20	0	40	15	20	20	40
Mean	11.66	18.33	15.83	36.66	12.5	17.5	14.16	37.5	11.66	17.5	12.5	35.83
std dev	9.309	9.309	8.010	2.581	10.36	8.803	11.14	2.738	9.309	8.803	9.874	2.041
std error	3.800	3.800	3.270	1.054	4.232	3.593	4.549	1.118	3.800	3.593	4.031	0.833

Table 5.2: Incline plane scores in saline control and quercetin treated animals in all treatment regimens before treatment (at 2 wks post surgery) and at the study end point (8 wks post surgery).

A plot of the incline plane values at eight weeks post surgery in all three treatment regimens is shown in Fig. 5.5.

Although increases in BBB were all together associated with increases in incline plane score no direct relation was found between BBB score point increases and the incline scale increases. It was observed for instance, that increase in BBB score by 1 to 3 points could bring about the same increase in incline plane values in some cases while they could vary in other cases. This could partly be explained by the non-uniform interval between the BBB score such that certain portions of the scale can occur more rapidly than others and may be affecting different motor pathways leading to different performances in BBB and incline plane test.

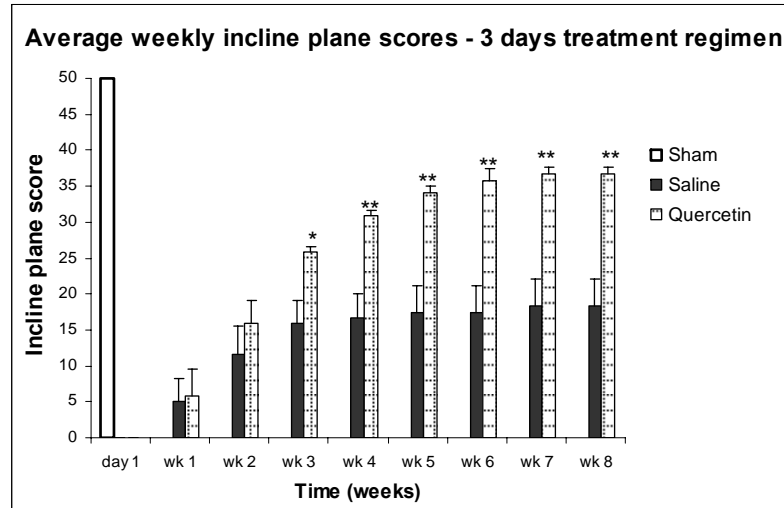


Fig 5.4 (a)

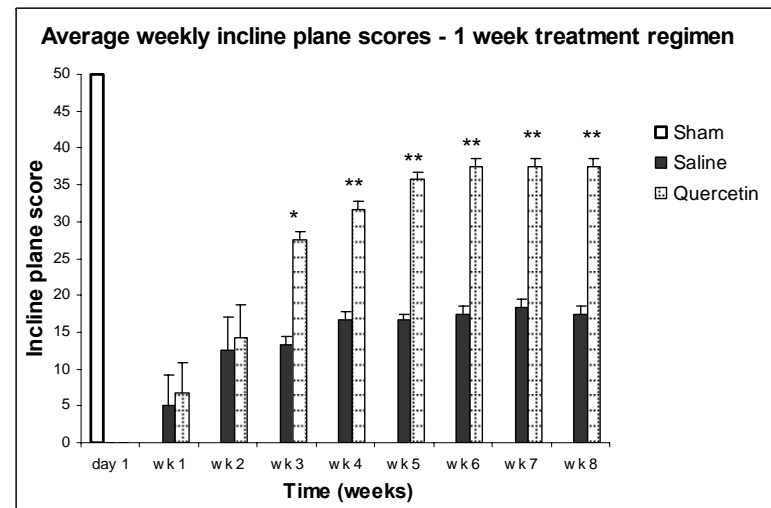


Fig 5.4 (b)

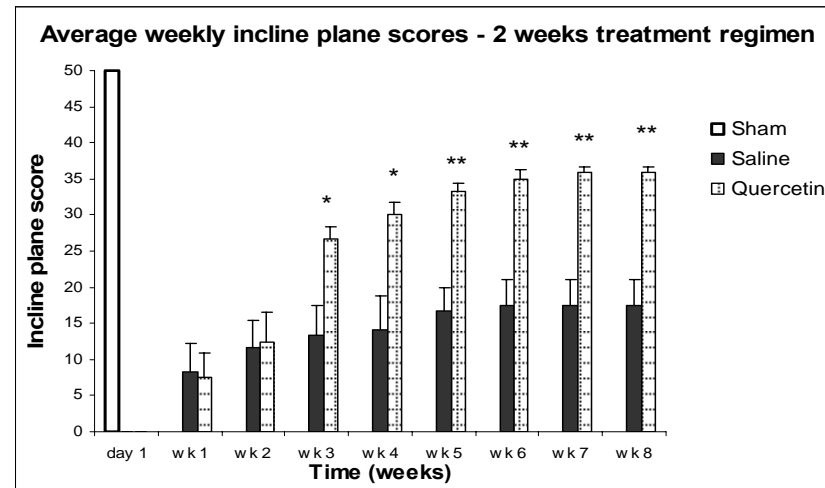


Fig 5.4 (c)

FIGURE 5.4 (a), (b), (c): Incline plane scores over the eight weeks study period in the 3 days (a), 1 week (b) and 2 weeks (c) treatment regimens. Sham animals all scored maximum throughout the study. The quercetin treated animals in all treatment regimens showed statistically significant increases in average incline plane scores by the first week following treatment (3 weeks post surgery) and reached a plateau by the third week post treatment (5 weeks post surgery). Values are expressed as mean \pm standard error (SE). * and **denotes significant difference between quercetin treated and saline control $p < 0.01$ and $p < 0.001$. $n = 6$ animals per group for each treatment regimen.

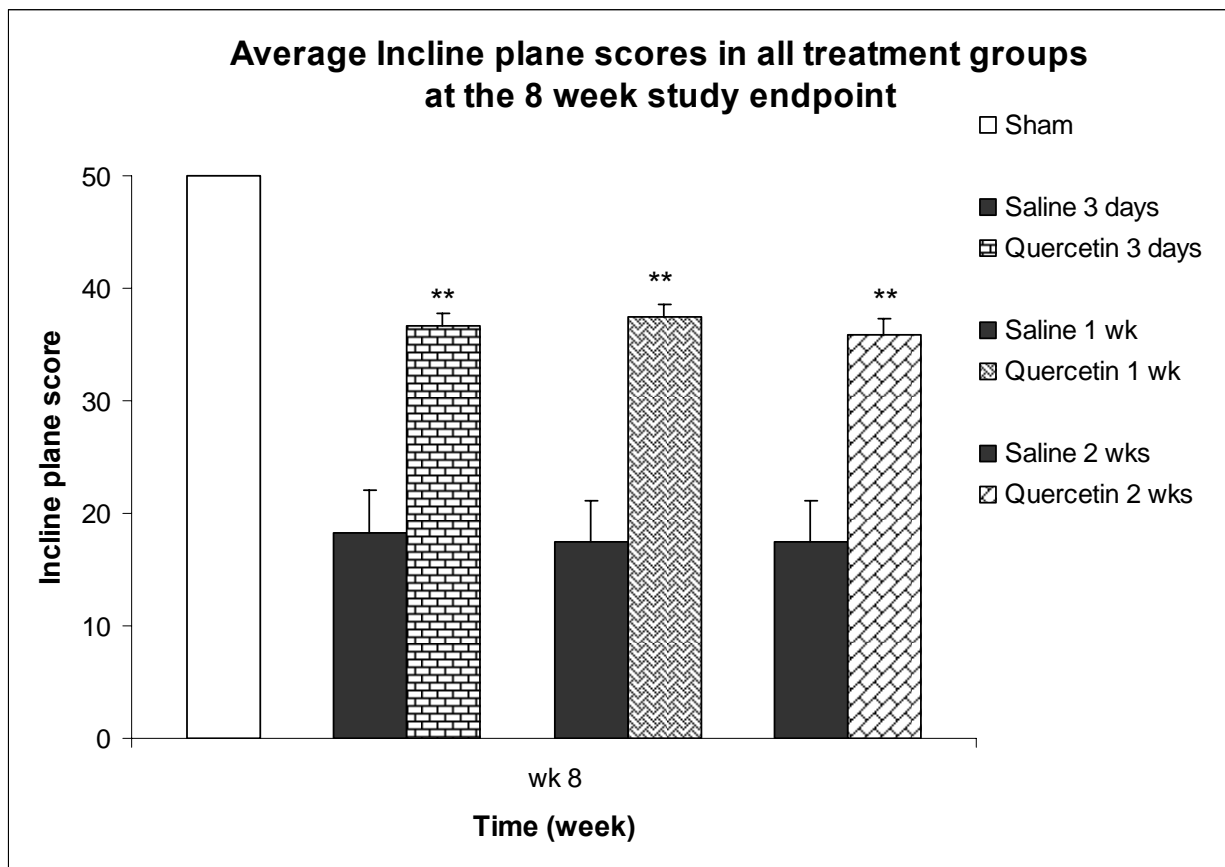


FIGURE 5.5: Incline plane score at the eight week study end point in the 3 days, 1 week and 2 weeks treatment regimens. Sham animals all show the maximum score. Quercetin treated all show significant score increase in all treatment regimen compared to saline controls. Values are expressed as mean \pm standard error (SE). **denotes statistically significant difference between quercetin treated and saline control at $p < 0.001$. $n=6$ animals per group for each treatment regimen.

5.2 Effect of quercetin on cell damage and tissue sparing following SCI

5.2.1 Indicators of damage

Gross assessment of tissue damage assessed by means of histology studies (H and E, LFB and TB) showed better preservation of tissue structure with less axon loss and associated cavity formation in quercetin treated compared to the saline controls. Analysis of spinal cord tissue collected at eight weeks following injury from saline control animals demonstrated a considerable loss of spinal cord tissue at the epicenter of the injury (Fig. 5.6). In the quercetin

treated animals, we observed smaller cystic cavity with better tissue preservation and organization. Microcystic cavities were observed in all tissue and mainly in the posterior white matter. In animals treated with quercetin, spared axons were observed more often in both gray and white matter whereas this was seldom the case in the saline control animals. The saline control animals had practically very little gray matter left at 8 weeks post-injury, compared to the quercetin treated.

5.2.2 Indicators of tissue sparing

LFB stained sections (Fig. 5.6) revealed normal, well organized myelinated appearance of white matter in all sham animals compared to the injured animals (saline controls and quercetin treated) that showed distortion of tissue architecture compared to the sham. However, compared to the saline control animals, the quercetin treated animals showed a better myelinated tract appearance indicative of better preservation of white matter content in the quercetin compared to the saline controls. Quantification of white matter in the injured (explained in method section 4.9.5) showed more white matter content in quercetin compared to saline suggesting more tissue sparing in the quercetin (n=6 per treatment regimen) compared to the saline controls (n=6 per treatment regimen). It should be noted that for each animal six sections (containing the epicentre of the injury) were analyzed for amount of LFB stained tissue. Since quantification of pixels captured from injured animals for each group were standardized to the uninjured (sham) animals, the sham animals all had the 100% score therefore their scores are shown only once in the graph in order to display the results more clearly for the reader and highlight the differences between treatments (i.e. quercetin and saline). A plot of the graph showing white matter quantification in various treatment regimens is shown in Fig. 5.7.

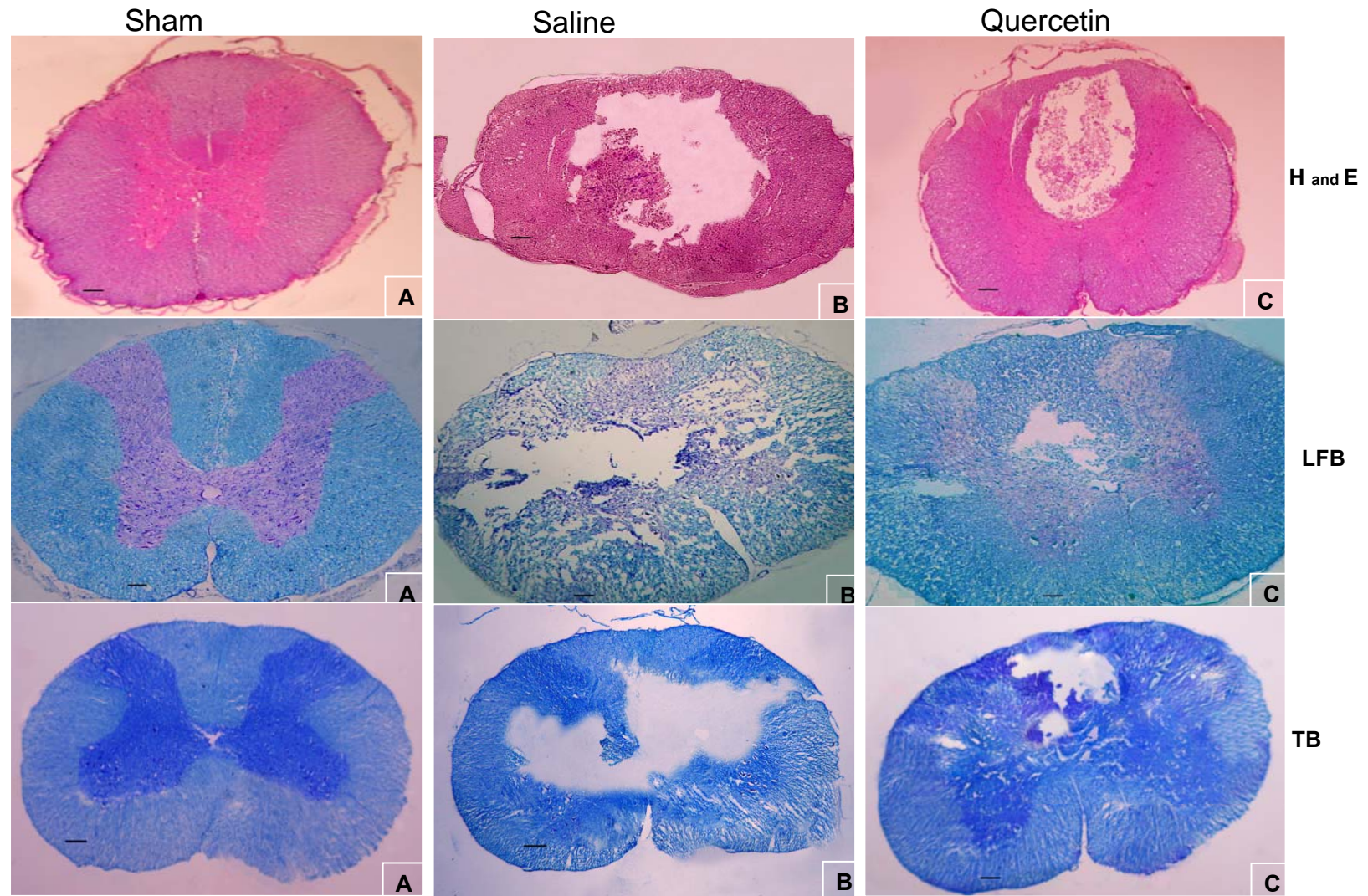


FIGURE 5.6 (a), (b), (c): Representative photomicrograph of spinal cord tissue from sham-operated A, saline control B, and quercetin treated C, from tissue harvested at eight weeks post surgery and stained with H and E, LFB and TB. Sham animals show gray and white matter with a normal appearance (original magnification $\times 10$). Scale bar = 100 μm .

Comparison of the results of LFB stained white matter quantification showed a statistically significant greater loss of white matter content in saline control compared to quercetin treated animals (21.81±0.93 vs. 38.19±1.5 white matter content in 3 days treatment regimen; 21.82±1.04 vs. 37.64±1.49 in 1 week treatment regimen; 21.82±1.83 vs. 37.91±0.72 in 2 weeks treatment regimen). Statistical analysis showed no statistically significant difference in outcome when the only quercetin treatment regimens were compared indicating that the duration of treatment had no statistically significant effect on the treatment outcome.

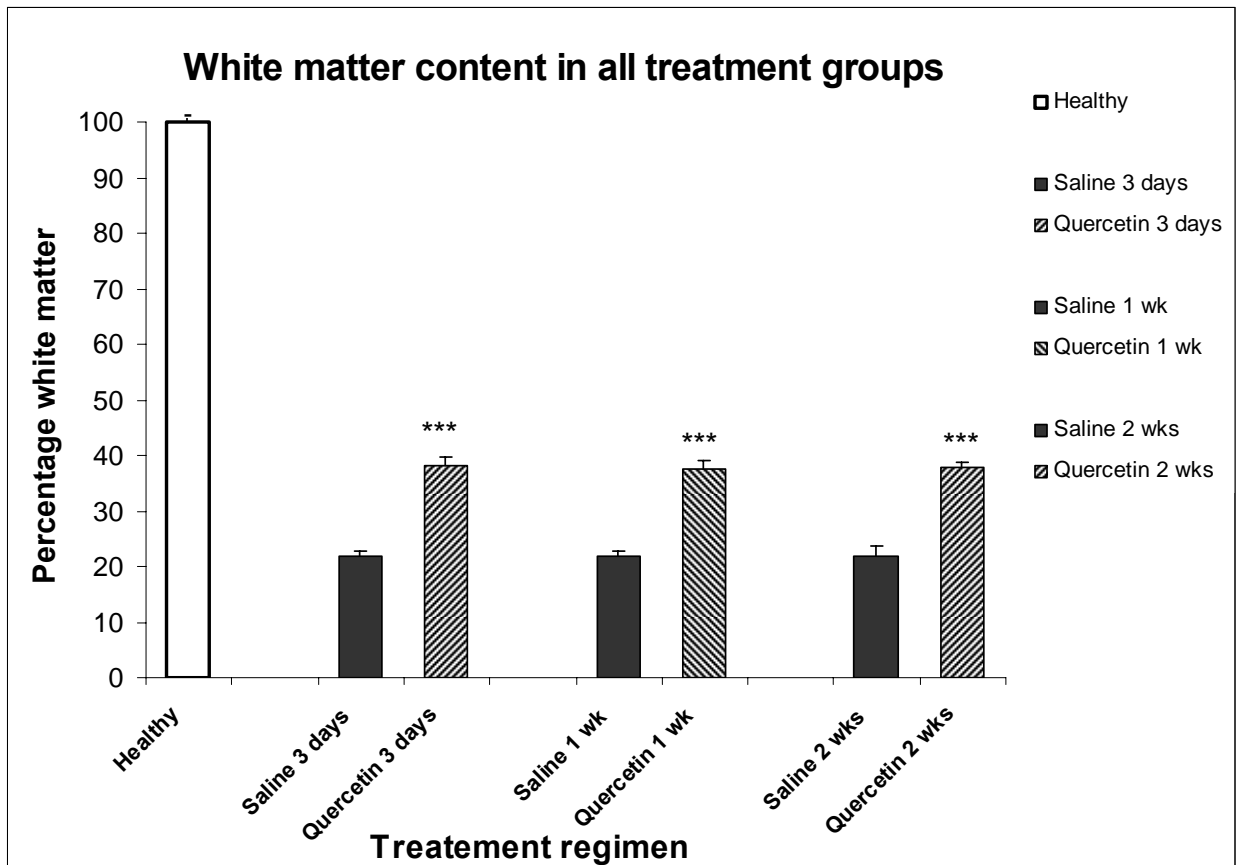


FIGURE 5.7: Graph shows white matter content from spinal cord harvested at the eight week study endpoint in all three treatment regimens (3 days, 1 week and 2 weeks). LFB stained sections containing the epicenter of the injury were quantified from six slides per animal. Healthy (uninjured) animals showed the maximum content. In injured animals (saline controls and quercetin treated), significantly more white matter content is observed in the quercetin treated than the saline controls indicative of more tissue sparing in quercetin treated regimens compared to saline controls. Values are expressed as mean ± standard error. ***denotes statistical significance at $p < 0.0001$. $n = 6$ animals per group for each treatment regimen.

The protective effect of delayed quercetin treatment in spinal cord injured rats is associated with decreased cell apoptosis.

6.1 Introduction

In the previous chapter we observed that even when administered at 2 weeks post surgery, quercetin promotes some functional recovery and this is associated with decreased gross cell structural damage and white matter sparing. The aim of this chapter is to assess tissue damage by measuring the extent of apoptosis using the TUNEL method complemented by immunocytochemistry and Western blots to detect caspase-3 activity and PARP cleavage.

6.2 Indicators of apoptosis

6.2.1 TUNEL staining:

Cytochemistry for DNA fragmentation with TUNEL method was done on the 3 days and 2 weeks treatment regimens. Both quercetin treated (n=6 per treatment regimen) and saline control (n=6 per treatment regimen) animals showed higher levels of apoptosis compared to the sham animals (n=6 per treatment regimen). However, more TUNEL positive signals were observed in tissue cross sections from saline control animals compared to quercetin treated (Fig. 6.1) indicative of more apoptotic cells in saline control animals compared to quercetin treated. In all cross sections examined, the bulk of TUNEL positive cell were observed in white matter compared to gray matter and the lateral and dorsal white matter (lateral and dorsal funuculi) were

more affected. In addition, a large number of positive cells were also observed in the tissue surrounding the cavity area. The average nuclei count for the total area selected for quantification (as evidenced with Hoechst stain) was approximately between 800-900 nuclei in the injured animals (i.e. injured saline control and quercetin treated) and ≥ 980 nuclei in the sham animals. Average TUNEL positive signals in saline control animals, ranged from between 70-90 positive cells per total area counted while in the quercetin treated the positive signals reduced to approximately between 40-60 positive signals positive cells per total area counted. Very few positive cells were observed in sham operated animals with an average of less than 3 positive cells per total area selected. Moreover, on visual observation the intensity of the positive signals in sham animals was less intense compared to those of the injured animals; suggesting that DNA fragmentation in sham animals was more tightly regulated and therefore more subtle than in the injured animals.

Comparison of the TUNEL positive signals from various treatment groups with ANOVA showed a highly significantly lower TUNEL signal expression in sham compared to the injured animals (i.e. both quercetin treated and saline control) in the two treatment regimens analyzed $p < 0.0001$ (only the 3 days and 2 weeks treatment regimen were analyzed for TUNEL counts). However amongst the injured animals, there was a significantly greater number of TUNEL positive cells in the saline control animals ($n=6$ per treatment regimen) compared to the quercetin treated animals ($n=6$ per treatment regimen) in all treatment regimens $p < 0.01$. ANOVA analyses showed no statistically significant differences between the quercetin treated animals in various regimens, indicating that the duration of treatment had no statistically significant effect on the treatment outcome. A graph of TUNEL positive signal counts in the 3 days and two weeks treatment regimens is shown in Fig. 6.2.

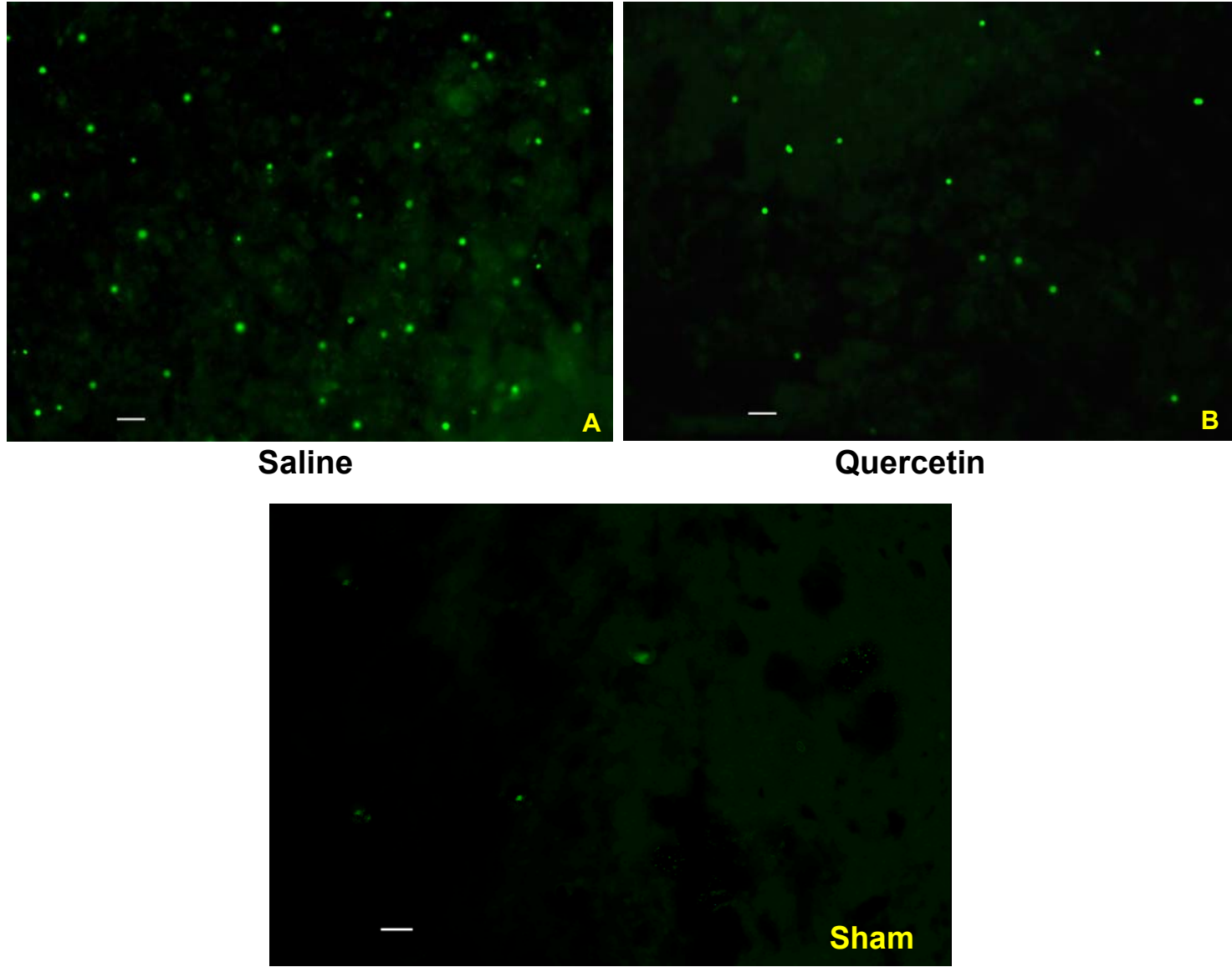


Fig. 6.1 (a)

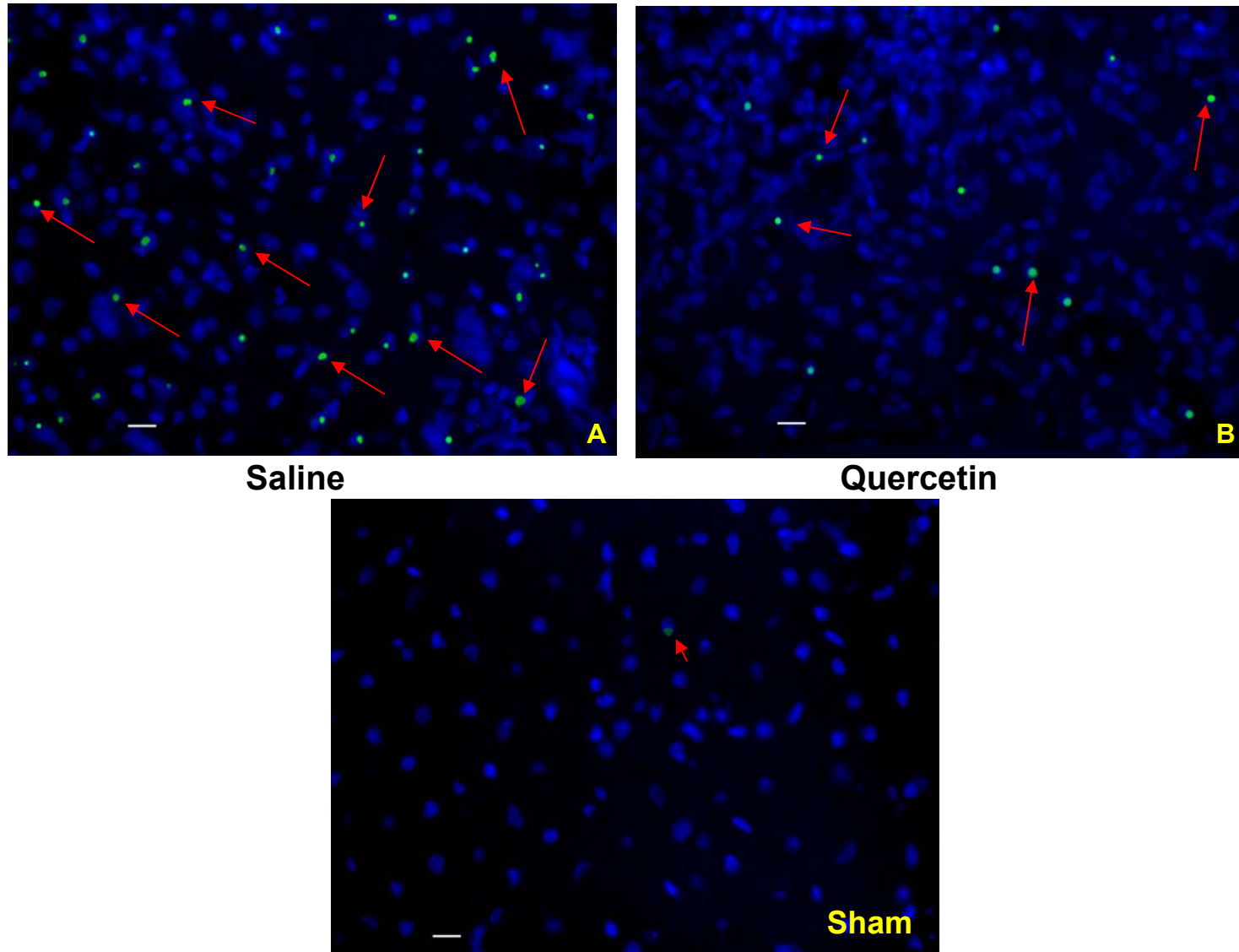
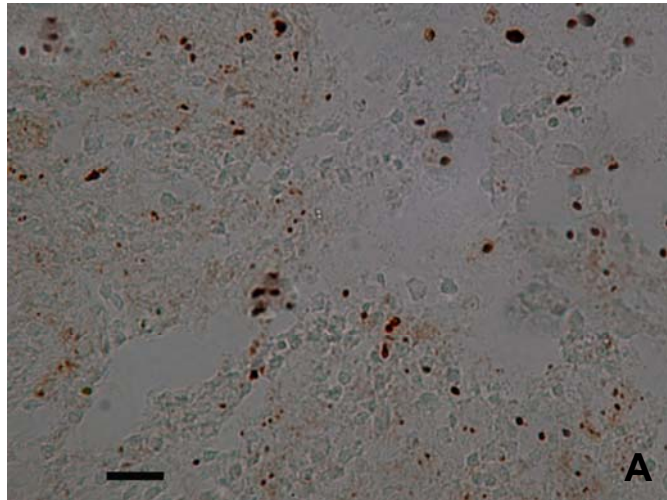
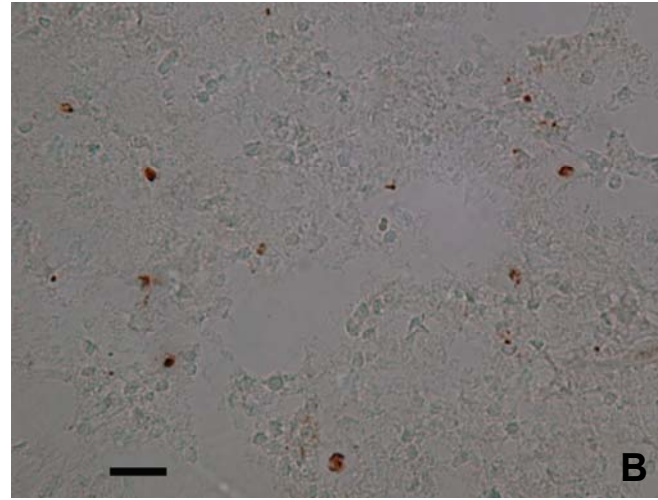


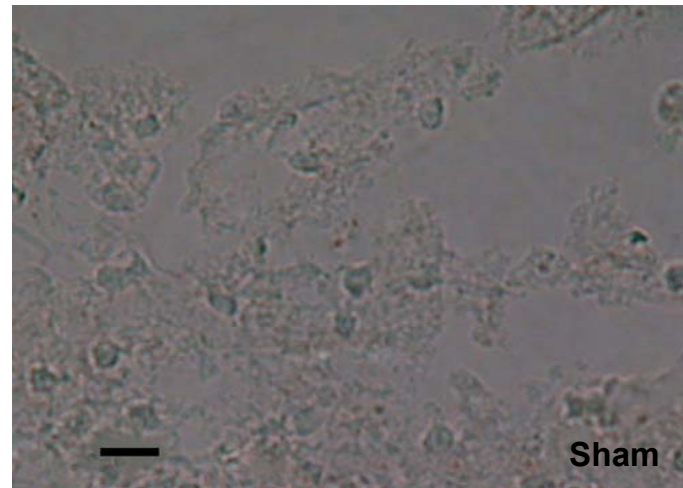
Fig. 6.1 (b)



Saline



Quercetin



Sham

Fig. 6.1 (c)

FIG 6.1 (a), (b), (c): Representative photomicrographs of TUNEL staining in saline control A, quercetin treated B, and sham operated, from tissue harvested at the eight week study endpoint. Fluorescent stained sections (a) show a greater number of TUNEL positive cells in the saline controls A, compared to quercetin treated B. Staining with Hoechst (b) allows visualization of nuclei in all cells; arrows (red) show TUNEL positive cells (green) with corresponding nuclei (stained blue). Sham animals showed minimal or no TUNEL positive signals. DAB stained sections (c) also show a greater number of TUNEL positive cells in the saline control A, compared to the quercetin-treated B and minimal signal in the sham. Scale bar = 50 μ m

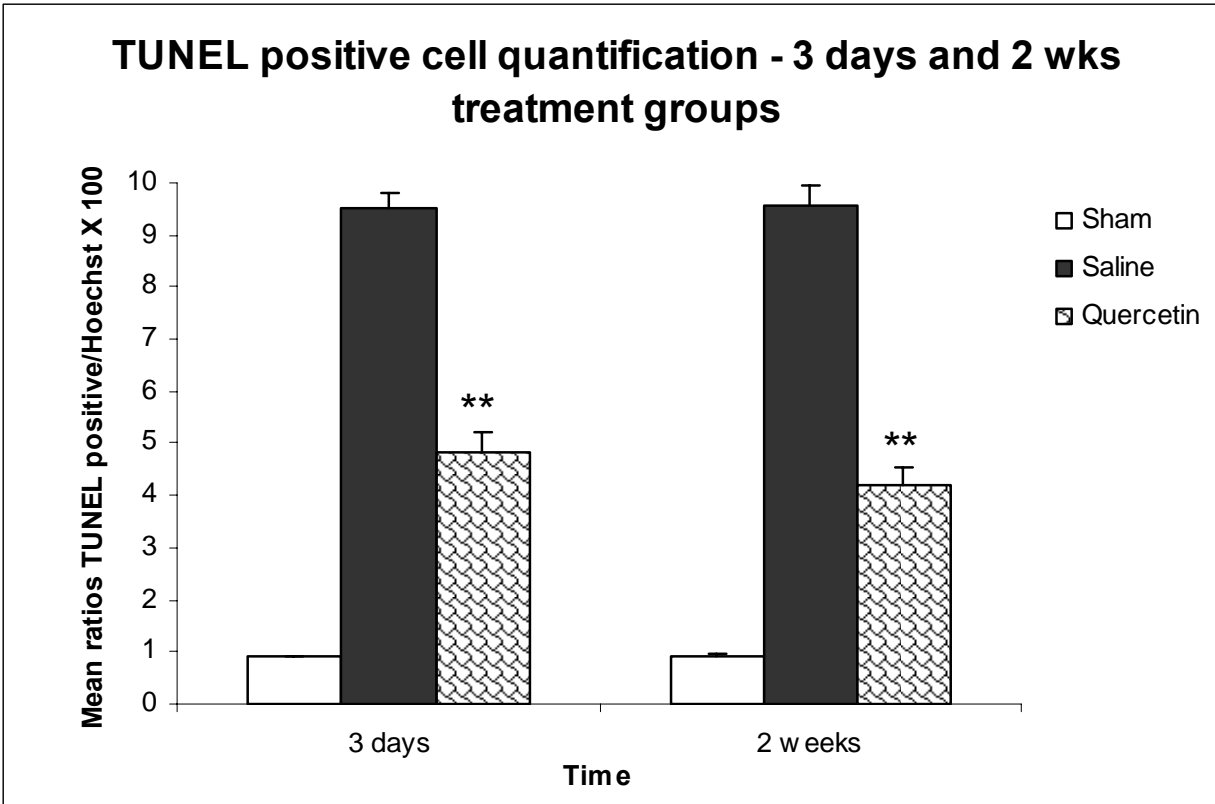


FIGURE 6.2: The graph shows TUNEL positive counts in the 3 days and two weeks treatment regimens, using sections from spinal cord tissues harvested at the eight week study endpoint. Positive cells within three pre-specified areas from tissue sections were counted from nine slides per animal (spaced ~1mm apart). Sham animals showed a minimal number of TUNEL positive cells. There were more TUNEL positive cells in both quercetin treated and saline controls animals compared to the sham animals but the number of TUNEL positive cells in the saline controls (n=6) was significantly higher than in the quercetin treated animals (n=6) indicative of more apoptotic cells in saline control animals compared to quercetin treated. Values are expressed as mean ratio \pm standard error. ** denotes statistically significant difference between quercetin treated and saline control at $p < 0.001$. n=6 animals per group for each treatment regimen.

6.2.2 Immunocytochemistry and Western blotting

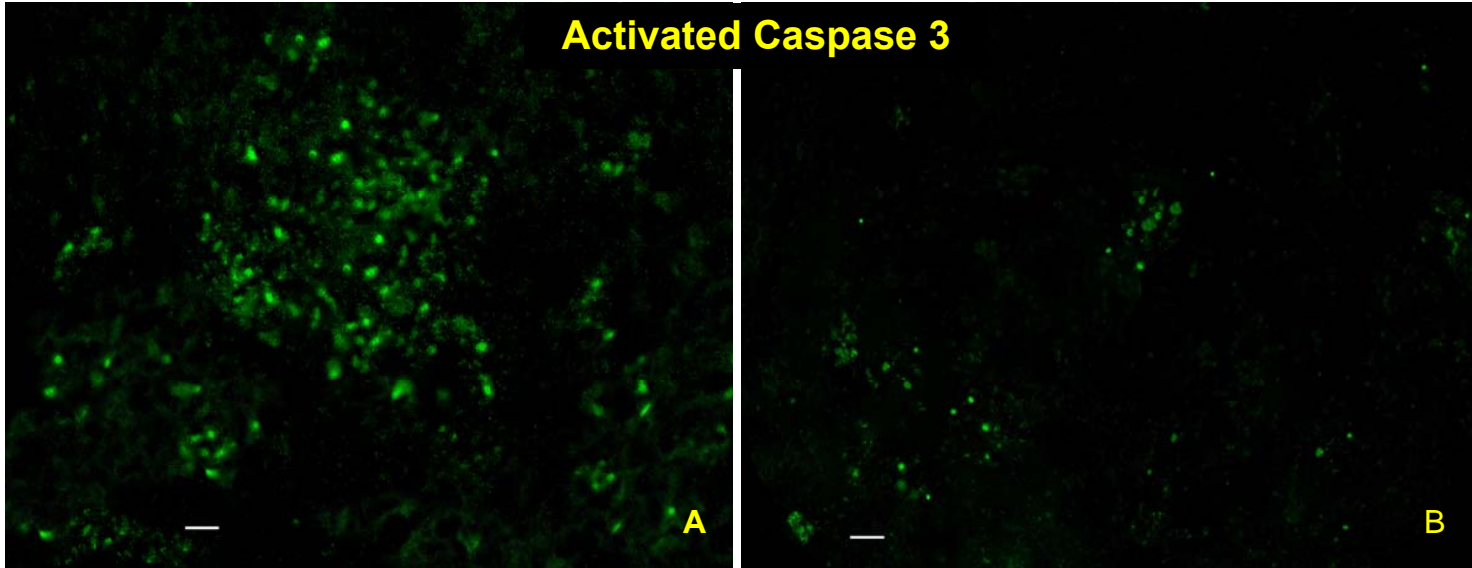
6.2.2.1 Activated caspase 3

Apoptosis is also characterized by activation of caspase-3. Visual examination of immunostained spinal cord sections and Western blot analyses of whole spinal cord tissues harvested at the eight weeks end point, showed increased caspase-3 activity in both quercetin treated (n=6 per treatment regimen) and saline control (n=6 per treatment regimen) animals compared to sham animals (n=6 per treatment regimen); indicating an increased activation of the

apoptotic process in all injured animals (i.e. quercetin treated and saline controls). However, observation of immuno stained slides (Fig. 6.3) and Western blot analysis of spinal cord (Fig. 6.4) for activated caspase-3 revealed a more intense expression of this enzyme in the saline control compared to quercetin treated animals in all treatment regimens; indicating a more profound apoptosis in the saline control compared to the quercetin treated animals.

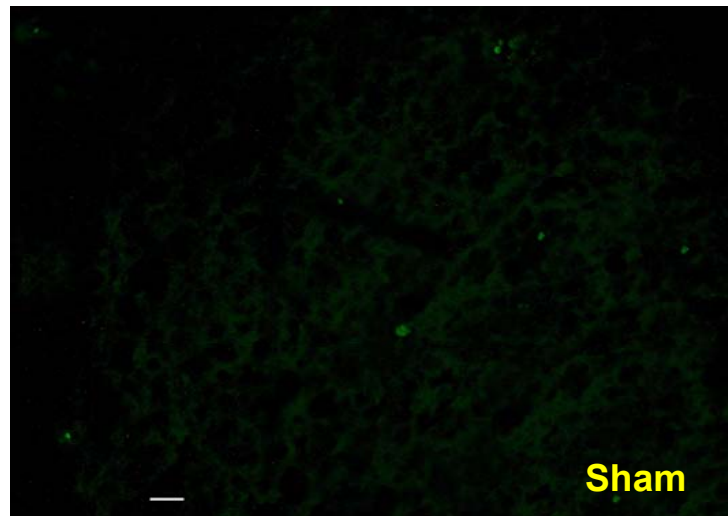
A plot of the intensity of bands developed from Western blot analyses of the 3 days and 2 weeks treatment regimen is shown in Fig. 6.4 (b). Analyses of the data using ANOVA demonstrated a significant increase in activated caspase 3 protein expression in both quercetin treated (n=6 per treatment regimen) and saline control (n=6 per treatment regimen) animals compared to the sham animals (n=6 per treatment regimen). However, there was a significantly higher caspase-3 activation in the saline control compared to the quercetin treated animals ($p<0.01$). These analyses showed no statistically significant difference between the quercetin treated groups in various regimens; indicating that the duration of treatment had no statistically significant effect on the treatment outcome.

Activated Caspase 3



Saline

Quercetin



Sham

Fig. 6.3 (a)

Activated caspase 3

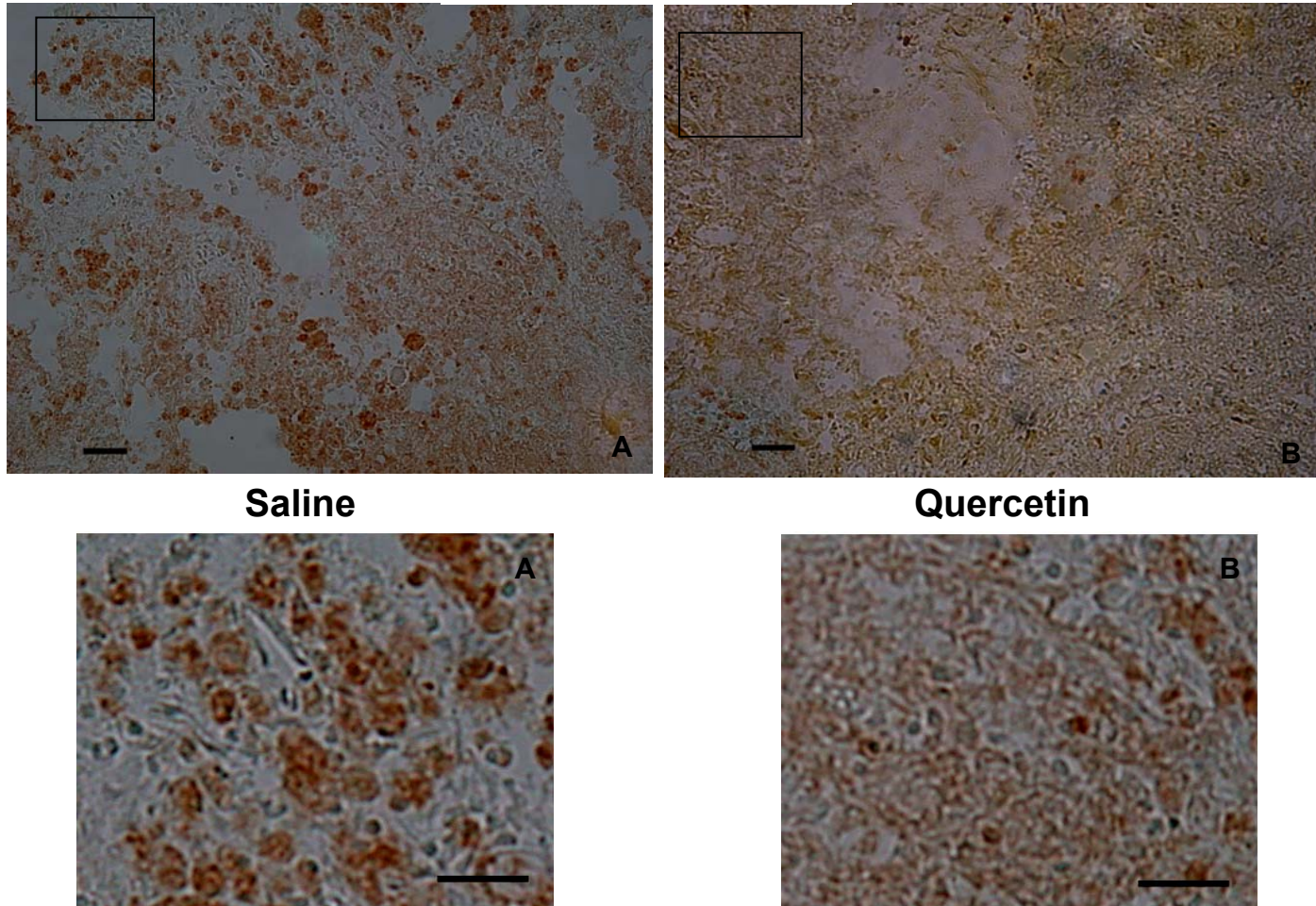


Fig. 6.3 (b)

FIGURE 6.3: Representative photomicrograph of spinal cord sections harvested at eight weeks post surgery and immuno-stained for activated caspase-3. Fluorescent (a) and DAB (b) immuno-stained sections show more activated caspase-3 signal in saline control A, compared to quercetin treated B, (scale bar =50 μ m). Sham animals show minimal fluorescent signal. The lower images in Fig. 6.3 (b), represent areas of intense signal (scale bar =20 μ m) selected from the respective images above (A and B) (scale bar = 50 μ m).

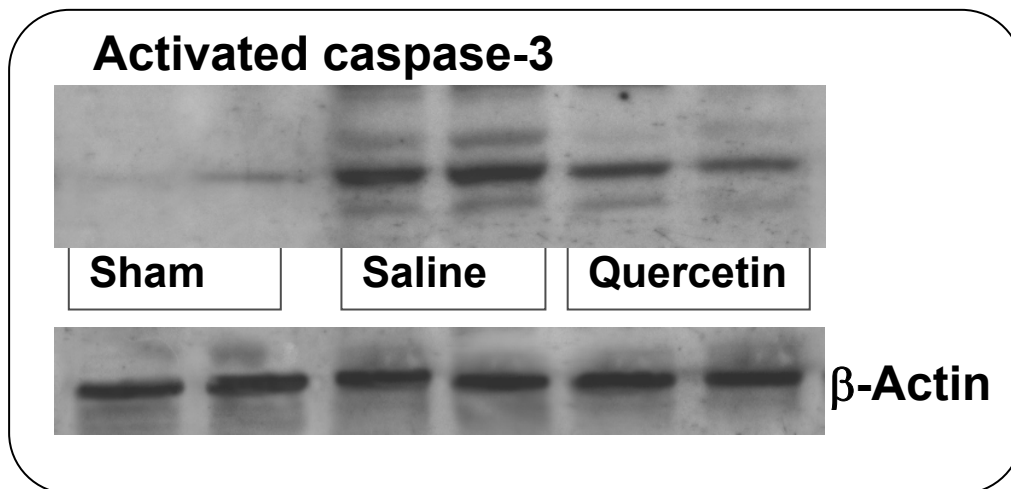


Fig 6.4 (a)

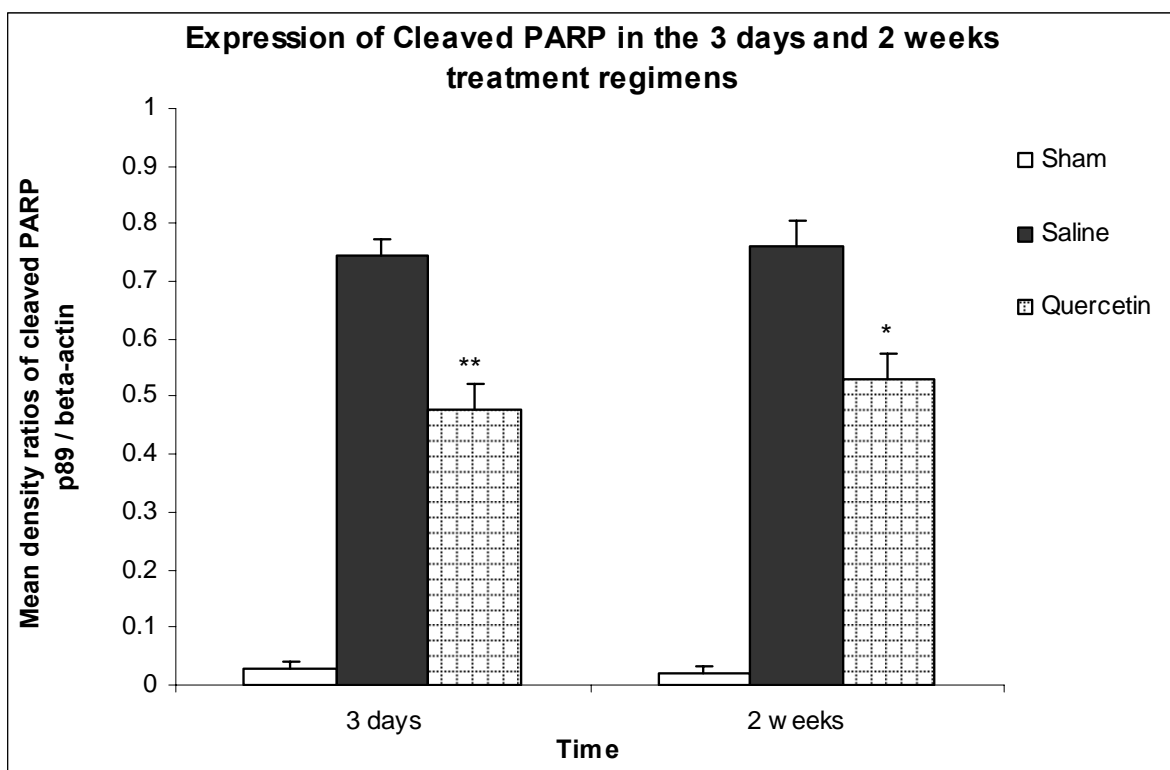


Fig 6.4b

FIGURE 6.4 (a) (b): Representative Western blot (a) and plot of band intensity (b) of activated caspase-3 expression in spinal cord tissue at eight weeks post surgery from sham saline control and quercetin treated animals. In all three treatment regimens, injured animals (quercetin treated and saline controls) show increased expression of caspase-3 activation compared to the sham animals (n=6); however, significantly greater expression of activated Caspase-3 is observed in saline controls (n=6) compared to quercetin treated (n=6). * and ** denotes a statistically significant difference between quercetin treated and saline control at $p < 0.01$ and $p < 0.001$. n=6 animals per group for each treatment regimen.

6.2.2.2 PARP cleavage

Proteolytic cleavage of PARP by activated caspase 3 is another hallmark of apoptosis. During apoptosis, endogenous full length PARP (116 kDa) is targeted by activated caspase 3 and cleaved to the large (89 kDa) and small (24 kDa) active fragments that further target other cellular element and eventually lead to the destruction of the cell.

The 3 days and 2 weeks treatment groups were analyzed for PARP cleavage. Similar to caspase-3 expression, both immunocytochemical analysis and western blot analysis showed increased PARP cleavage in both quercetin treated (n=6 per treatment regimen) and saline control (n=6 per treatment regimen) animals compared to the sham (n=6 per treatment regimen) animals in all treatment regimen analyzed. Amongst the injured animals (quercetin treated and saline controls), the saline controls had significantly higher increases in PARP cleavage compared to the quercetin treated animals. Visual examination of spinal cord sections immunostained for cleaved PARP revealed a stronger signal in the saline control animals compared to the quercetin treated (Fig. 6.5). Western blotting analysis with anti-PARP polyclonal antibody confirmed increased expression of cleaved-PARP fragments (89 and 24 kDa) in saline control animals compared to quercetin treated (Fig. 6.6a). A higher expression of the full length (116 kDa) PARP (uncleaved) was seen in the quercetin treated compared to the saline controls. Quantification of the Western blot band intensity of the larger (89 kDa) PARP fragment and analyses of the data with ANOVA showed a significantly higher expression of the protein in the saline control compared to the quercetin treated animals ($p < 0.01$) (Fig 6.6b). Results showed no statistically significant difference between the quercetin treated groups in various regimens; indicating that the duration of treatment had no statistically significant effect on the treatment outcome.

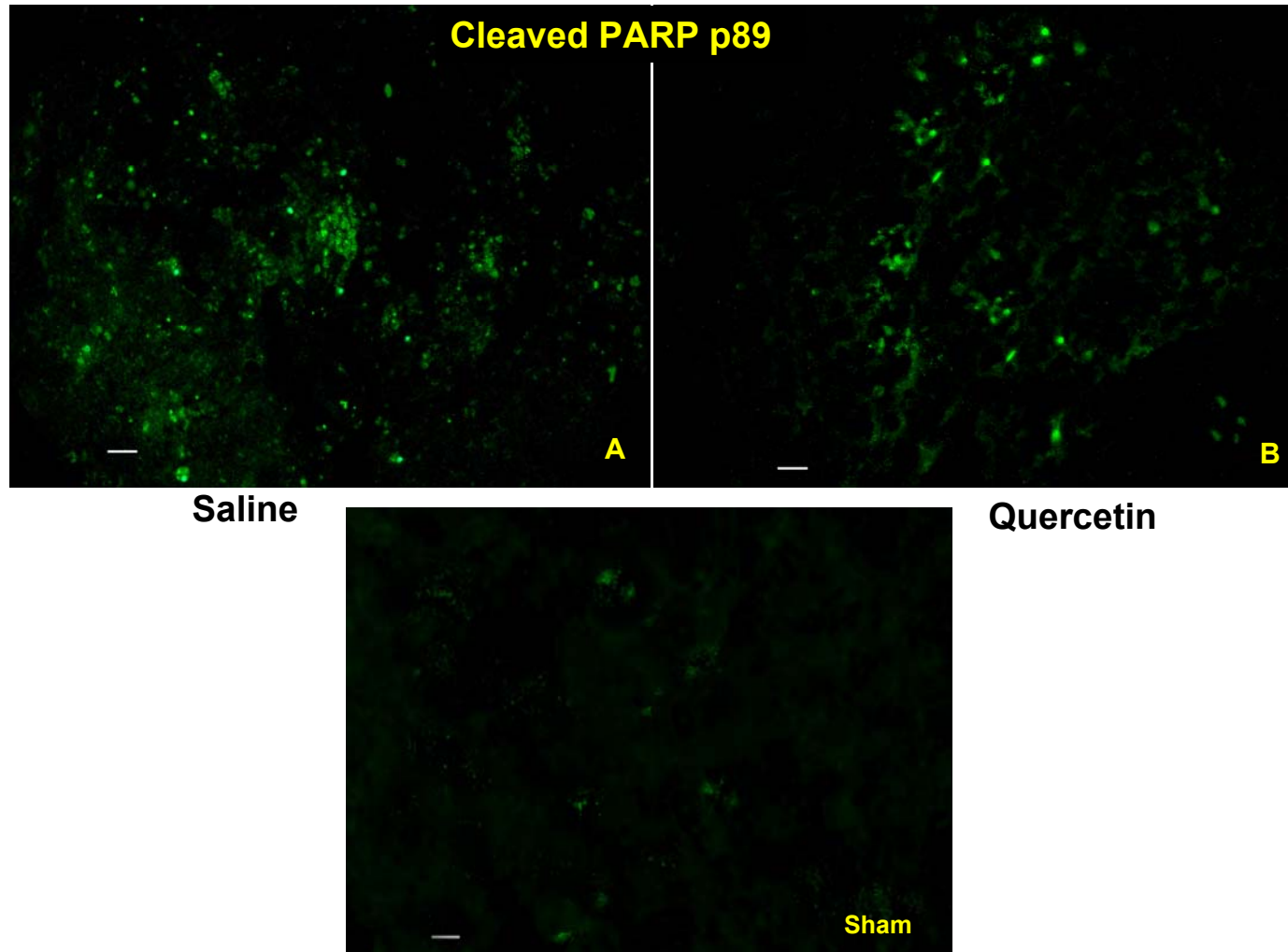


FIGURE 6.5: Representative photomicrograph of fluorescent immunocytochemical staining for cleaved PARP another hallmark of apoptosis in the saline control A, quercetin treated B and sham. Sections were obtained from spinal cord tissues harvested at the eight week study endpoint. Increased PARP expression is observed in all the injured animals (saline control and quercetin treated) but there are more positive cells with more pronounced expression of cleaved PARP fragment (p89) in saline controls A, compared to the quercetin treated B. Sections from sham animals show minimal fluorescent signal. Scale bar = 50 μm

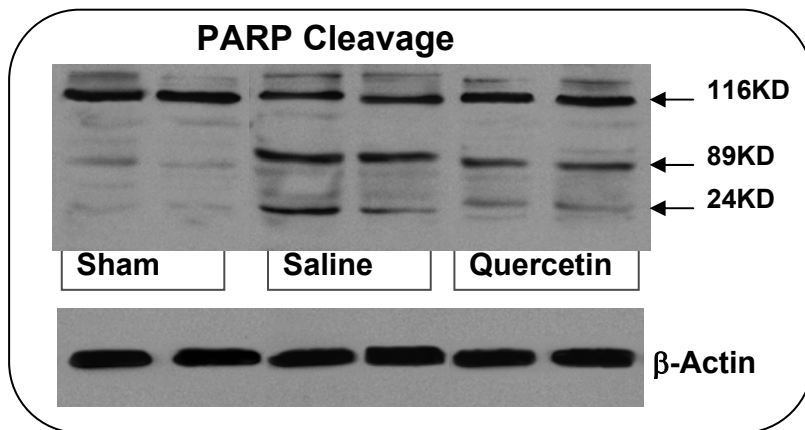


Fig. 6.6 (b)

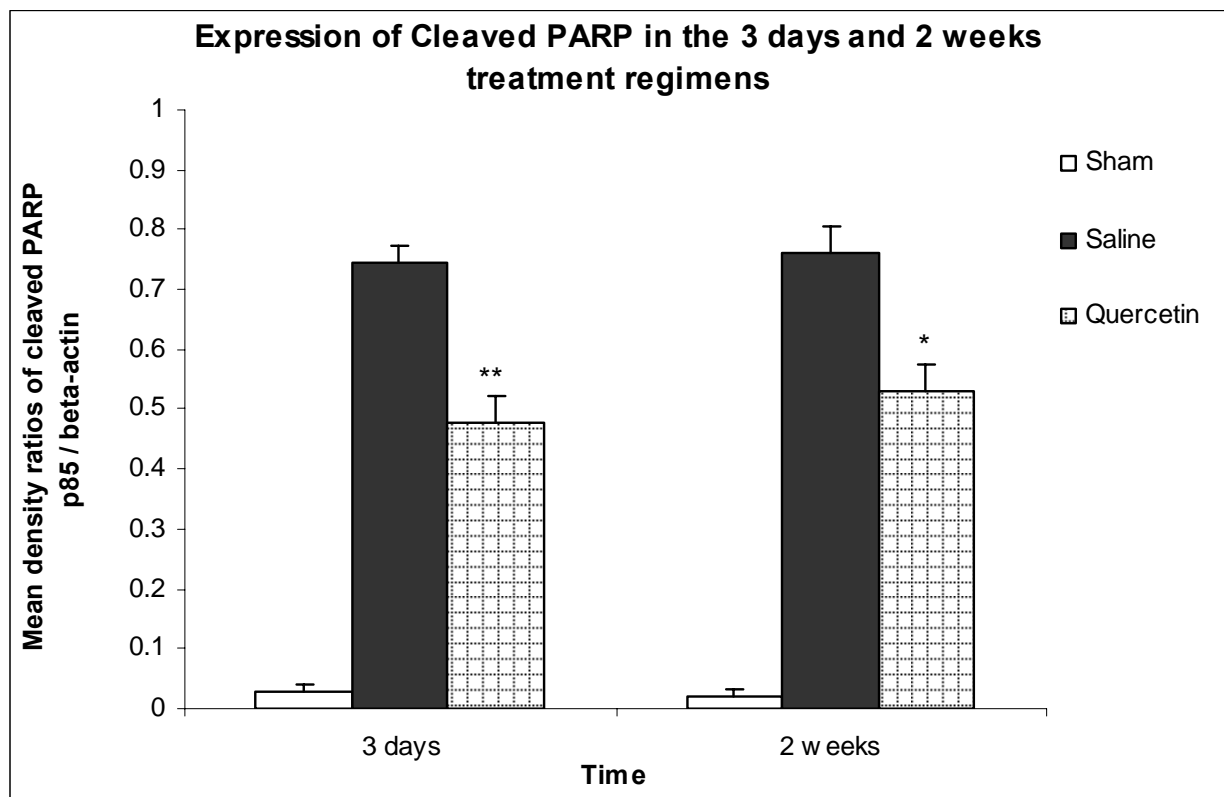


Fig. 6.6 (b)

FIGURE 6.6 (a) and (b): Representative Western blot (a) for PARP expression in sham, saline and quercetin treated animals harvested at eight weeks post surgery. In all three treatment regimens, injured animals (quercetin treated and saline controls) show increased expression of cleaved PARP (89 and 24kD) compared to the sham animals (n=6); however, more PARP cleavage is seen in the saline control (n=6) with more intense expression of cleaved fragment (89 and 24 kDa) compared to the quercetin treated (n=6). Graph (b) shows a plot of the intensity of the Western blot bands for cleaved PARP (large 89 kDa fragment) in the sham, saline control and quercetin treated spinal cord of animals from the 3 days and 2 weeks treatment regimen. Values are expressed as mean density ratio \pm standard error. * and ** denotes statistically significant difference between quercetin treated and saline control at $p < 0.01$ and 0.001 . n=6 animals per group for each treatment regimen.

Delayed quercetin treatment in traumatic spinal cord injury improves axon function, inhibits excess inflammatory response and inhibits phosphorylation of various MAPKs.

7.1 Indicator of improvement of axoplasmic function

7.1.1 β -amyloid precursor protein (β -APP) accumulation:

One protein that is transported via axoplasmic transport is the β amyloid precursor protein (β -APP); hence, axoplasmic transport impairment can be noted by accumulations of β -APP. Visual analysis of the spinal cord sections that were immunocytochemically stained for β -APP and Western blotting analyses of whole spinal cord from tissues harvested at the eight week study end point, showed increased accumulation of β -APP in all injured animals (i.e. saline controls and quercetin treated) compared to the sham animals. However, the saline control animals (n=6 per treatment regimen) showed more accumulation of the protein compared to the quercetin treated animals (n=6 per treatment regimen) (Fig. 7.1 and 7.2), indicating an improvement of axoplasmic transport in the quercetin treated compared to the saline control animals. These analyses were done on spinal cord tissues harvested from the 3 days and 2 weeks treatment regimens. Results the quantification of Western blot bands and statistical analysis of these data show a significant increase in β -APP accumulation in the saline control animals (n=6 per treatment regimen) compared to the quercetin treated animals (n=6 per treatment regimen) $p < 0.01$. A plot of the intensity of the bands from scanned Western blot is shown in Fig. 7.2 b.

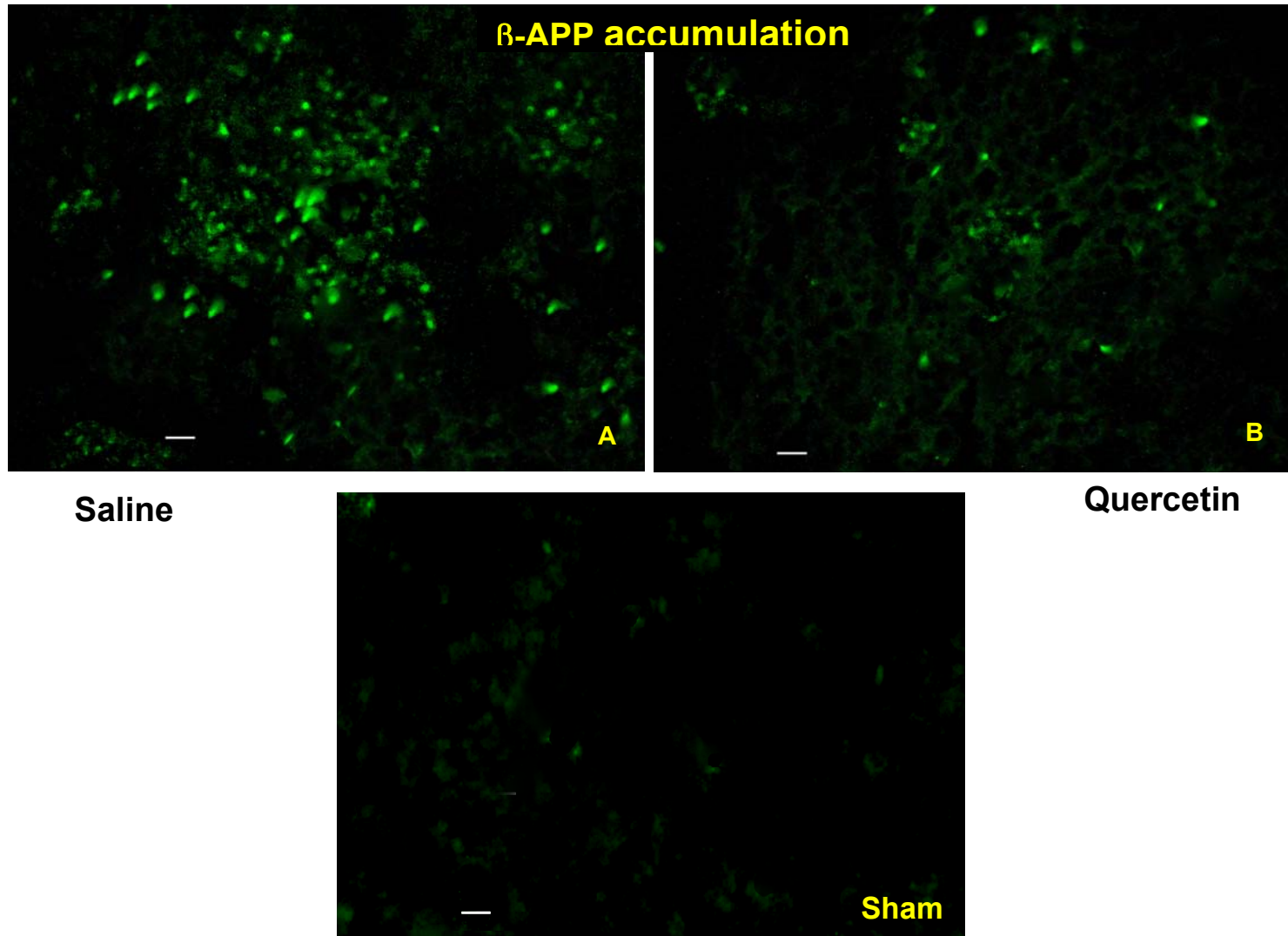


FIGURE 7.1: Representative photomicrograph of β -APP fluorescent immuno-stained sections in saline control A, quercetin treated B, and sham operated using sections from spinal cords harvested at eight weeks post surgery. There is lesser β -APP signal in the quercetin treated compared to the saline controls indicative of better axon function in the quercetin treated. Sections from sham animals show minimal signal. Scale bar = 50 μ m

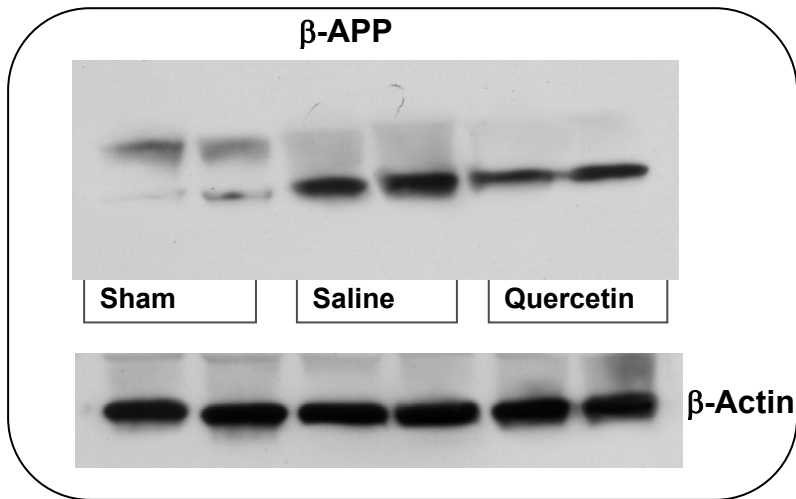


Fig.7.2 (a)

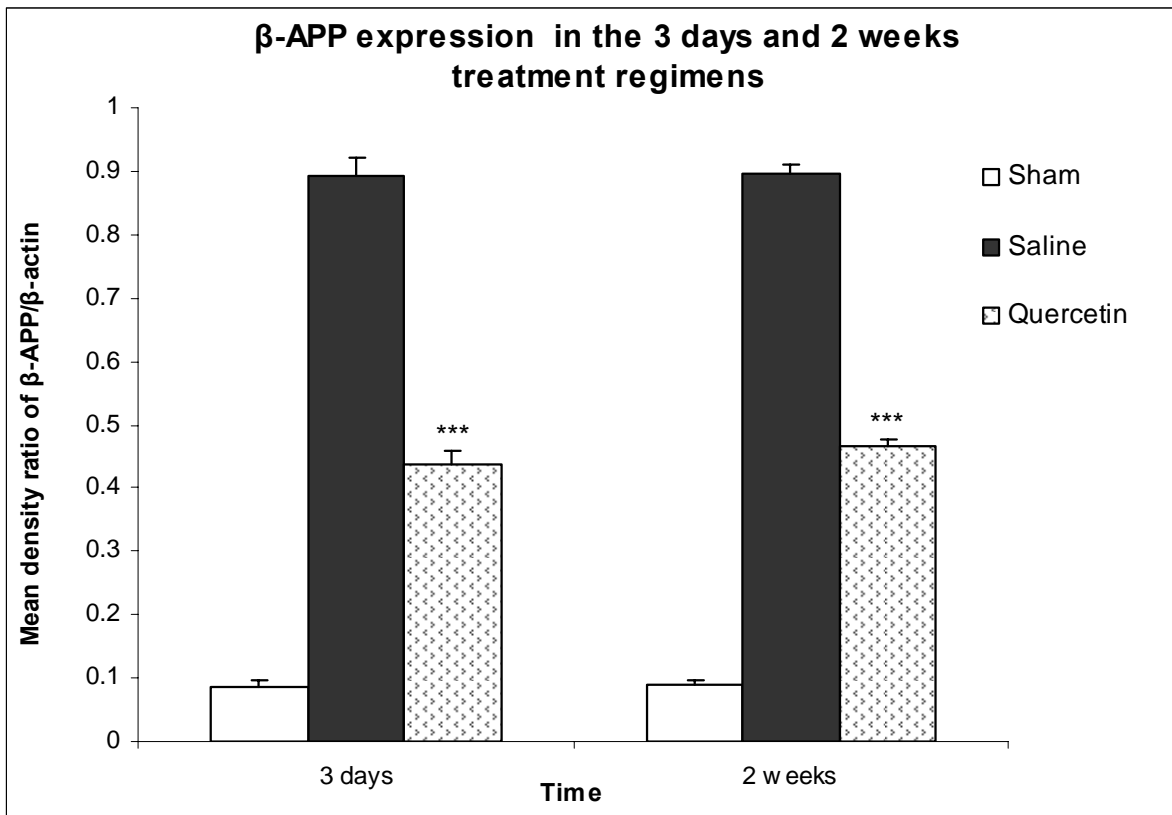


Fig.7.2 (b)

FIGURE 7.2 (a) (b): Representative Western blot (a) of β -APP accumulation at eight weeks post surgery in sham, saline and quercetin treated animals. A plot of the intensity of the bands from Western blots (b) shows increased expression of β -APP in both saline controls and quercetin treated compared to the sham animals; however, this increase β -APP expression is of a significantly greater intensity in the saline controls (n=6) compared to the quercetin treated (n=6). Values are expressed as mean density ratio \pm standard error. *** denotes a statistically significant difference between quercetin treated and saline control at $p < 0.0001$. n=6 animals per group for each treatment regimen.

No statistically significant difference was found when the quercetin treated animals in various regimens were compared; indicating that the duration of the treatment had no statistically significant effect on the treatment outcome.

7.2 Indicators of Reactive gliosis and inflammation

7.2.1 Glial Fibrillary Acidic Protein (GFAP)

Increased GFAP is a marker for astrogliosis. On examination of spinal cord sections (harvested at eight week study end point) that were immuno-stained for the GFAP epitopes, we observed that the sham animals visually exhibited basal levels of GFAP expression manifested by delicate-looking astrocytes with relatively small cell bodies and thin projections. Increased expression of GFAP and increased cell body size causing hypertrophy was observed in both injured saline control (n= 6 per treatment regimen) and quercetin treated (n= 6 per treatment regimen) compared to the sham controls. However, quercetin administration resulted in GFAP positive cells with smaller cell bodies and thinner projections compared to the control animals that received only saline vehicle (Fig. 7.3). In addition, Western blot analyses of the tissue homogenates confirmed the increased GFAP expression in all injured animals (saline controls and quercetin treated) with a significantly greater increase in the saline control (n= 6 per treatment regimen) animals compared to the quercetin treated (n= 6 per treatment regimen) (Fig. 7.4a).

Tissues were analyzed from two treatment regimens (i.e. 3 days and 2 weeks quercetin treatment).

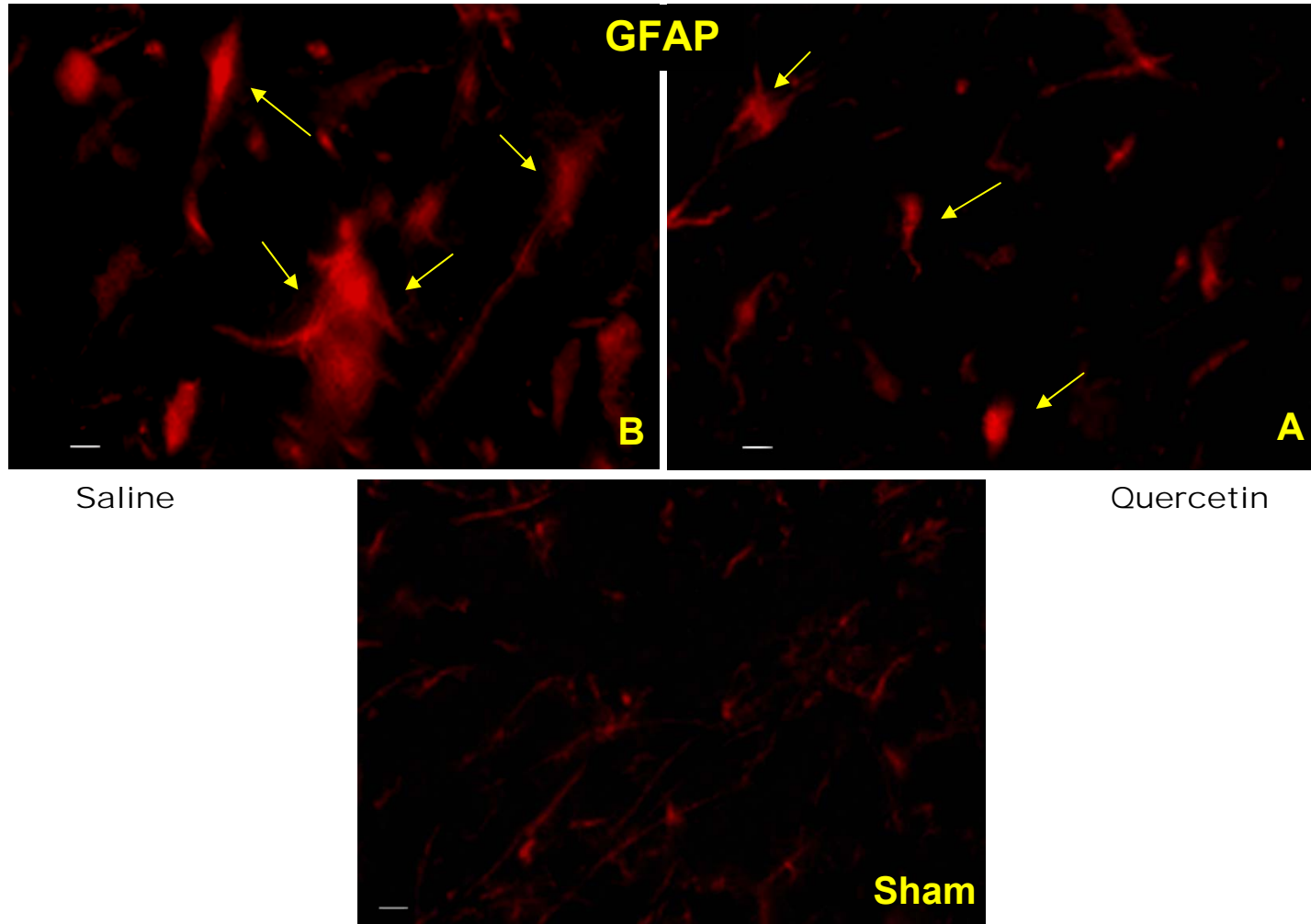


FIGURE 7.3: Representative photomicrograph of GFAP immunoreactivity in saline A, quercetin treated B and sham operated, using sections from spinal cords harvested at eight weeks post surgery. Reactive gliosis is manifested by an increased number of glial fibrillary acidic protein (GFAP) positive cells and content, accompanied by cell body hypertrophy (arrow). Saline control sections show more positive cells and more intense GFAP signal compared to quercetin treated indicative more astrogliosis in saline as compared to quercetin treated. The sham animals show basal levels of GFAP expression demonstrated by astrocytes with relatively small cell bodies and thin projections. Scale bar = 50 μ m

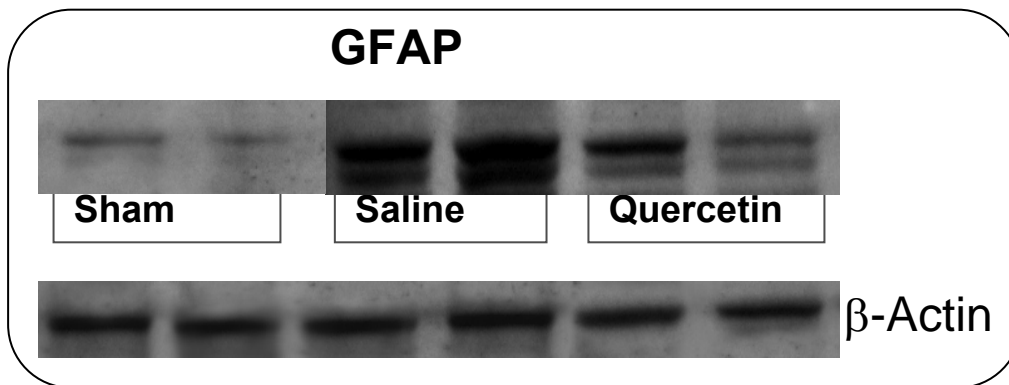


Fig 7.4a

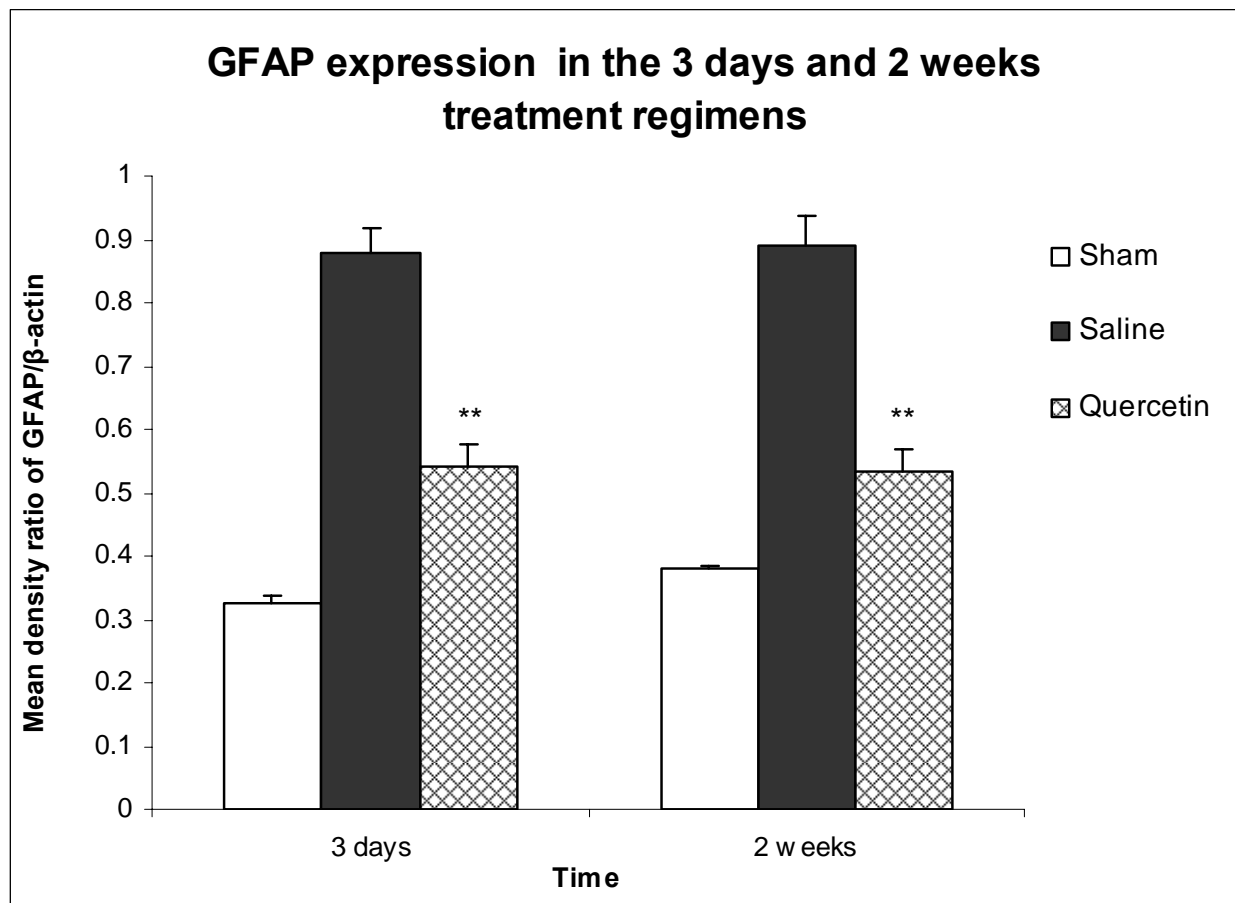


Fig 7.4b

FIGURE 7.4 (a) (b): Representative Western blot (a) of spinal cord at eight weeks post surgery from sham saline and quercetin treated animals. Increased expression of GFAP is observed in both saline controls (n=6) and quercetin treated (n=6) compared to the sham animals (n=6) but there is significantly higher GFAP expression in saline controls compared to quercetin treated. A plot (b) of the intensity of the bands from Western blots in two treatment regimens (i.e. 3 days and 2 weeks) showed a statistically significant increase in the saline control compared to the quercetin treated. Values are expressed as mean density ratio \pm standard error. ** denotes statistically significant difference between quercetin treated and saline control at $p < 0.001$. n=6 animals per group for each treatment regimen.

Analysis of variance of the Western blot data demonstrated a significantly greater increase in GFAP expression in saline control (n=6 per treatment regimen) compared to quercetin treated animals (n=6 per treatment regimen) ($p < 0.01$). No statistically significant difference was found between the quercetin treated groups in various treatment regimens; indicating that the duration of treatment in each regimen had no statistically significant effect on the final outcome. A plot of the intensity of bands from scanned Western blots is shown in figure 7.4b.

7.2.2 ED1 expression

ED1 is a marker for activated microglia and macrophages. Spinal cord sections immunostained for ED1 (microglia) visually showed increase immunoreactivity in all injured animals (i.e. quercetin treated and saline controls) compared to the sham animal but the saline control animals showed more intense signals compared to the quercetin treated (Fig. 7.5). These observations were also confirmed by Western blotting analyses (Fig 7.6a). Tissues were analyzed from two treatment regimens (i.e. 3 days and 2 weeks quercetin treatment).

Quantification of the intensity of bands from scanned Western blots and comparison of the results with ANOVA showed a significant increase of ED1 expression in the saline control animals (n=6 per treatment regimen) compared to quercetin treated animals (n=6 per treatment regimen) within each treatment regimen. However, results show no statistically significant difference between quercetin treated groups in various treatment regimens; indicating that the duration of the treatment had no effect on the final outcome.

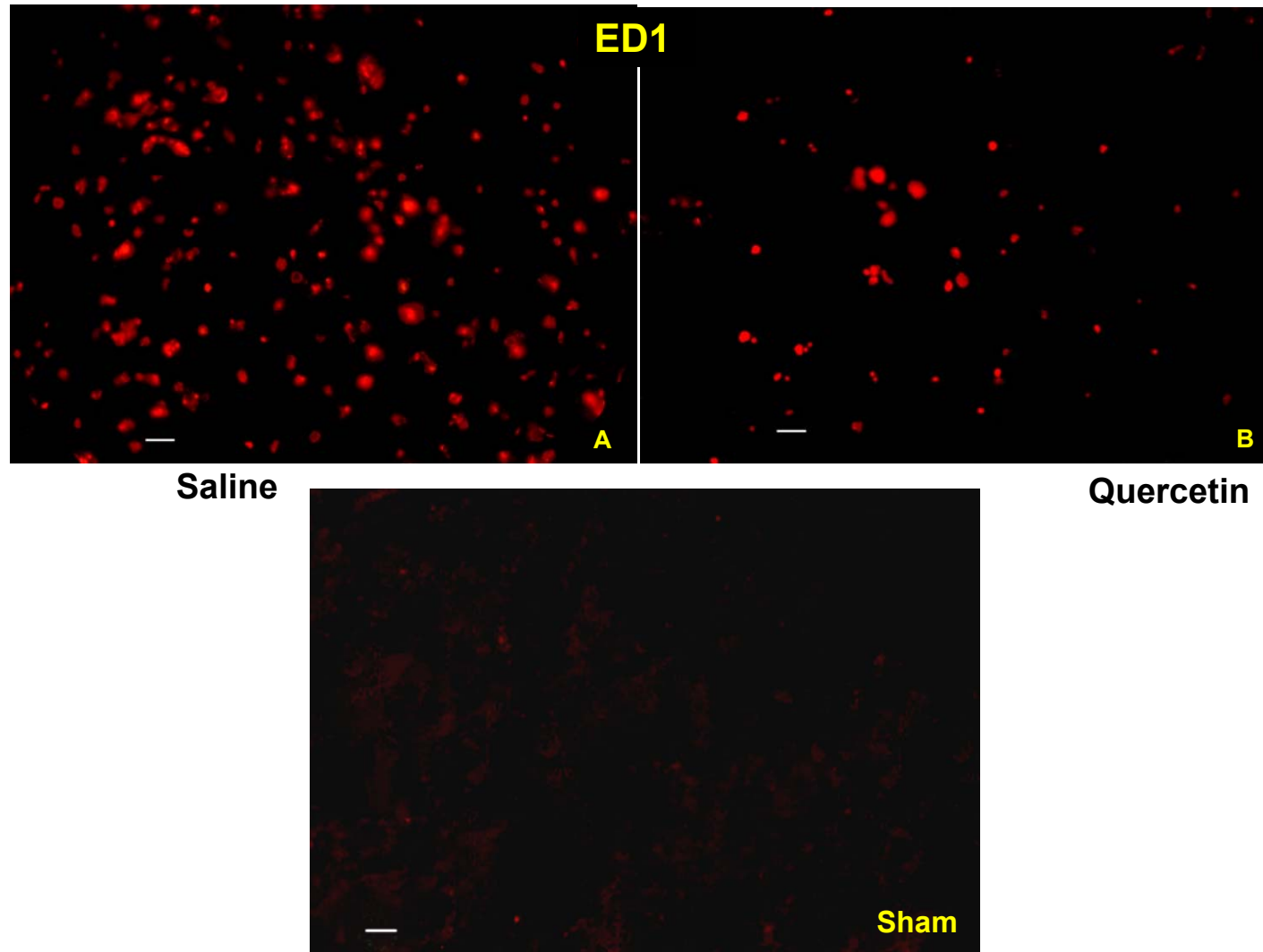


FIGURE 7.5: Representative photomicrograph of ED1 immunoreactivity in saline control A, quercetin treated B, and sham operated, using sections from spinal cords harvested at eight weeks post surgery. Increased ED1 expression is observed in both saline controls and quercetin treated compared to the sham animals; however, the saline control sections show more intense ED1 signal compared to quercetin treated indicative of more activated microglia in the saline compared to the quercetin treated. Sham sections show minimal ED1 immunoreactivity. Micrometer bar = 50 μ m.

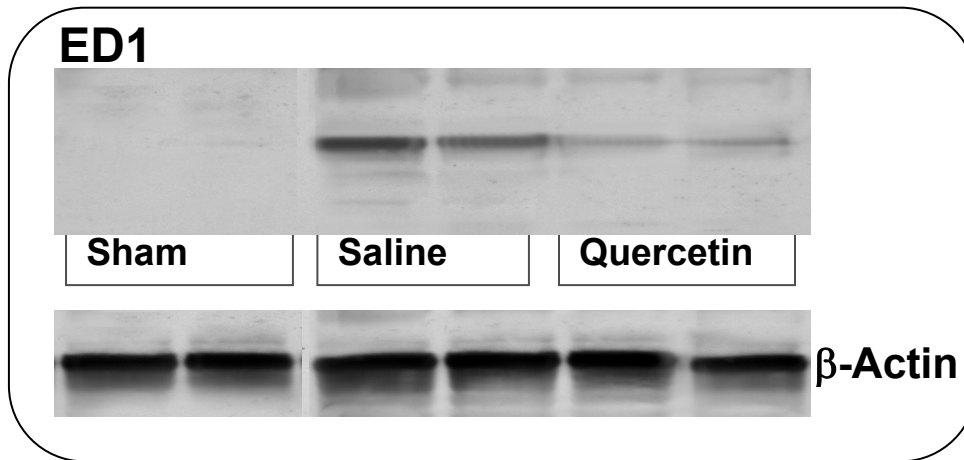


Fig 7.6b

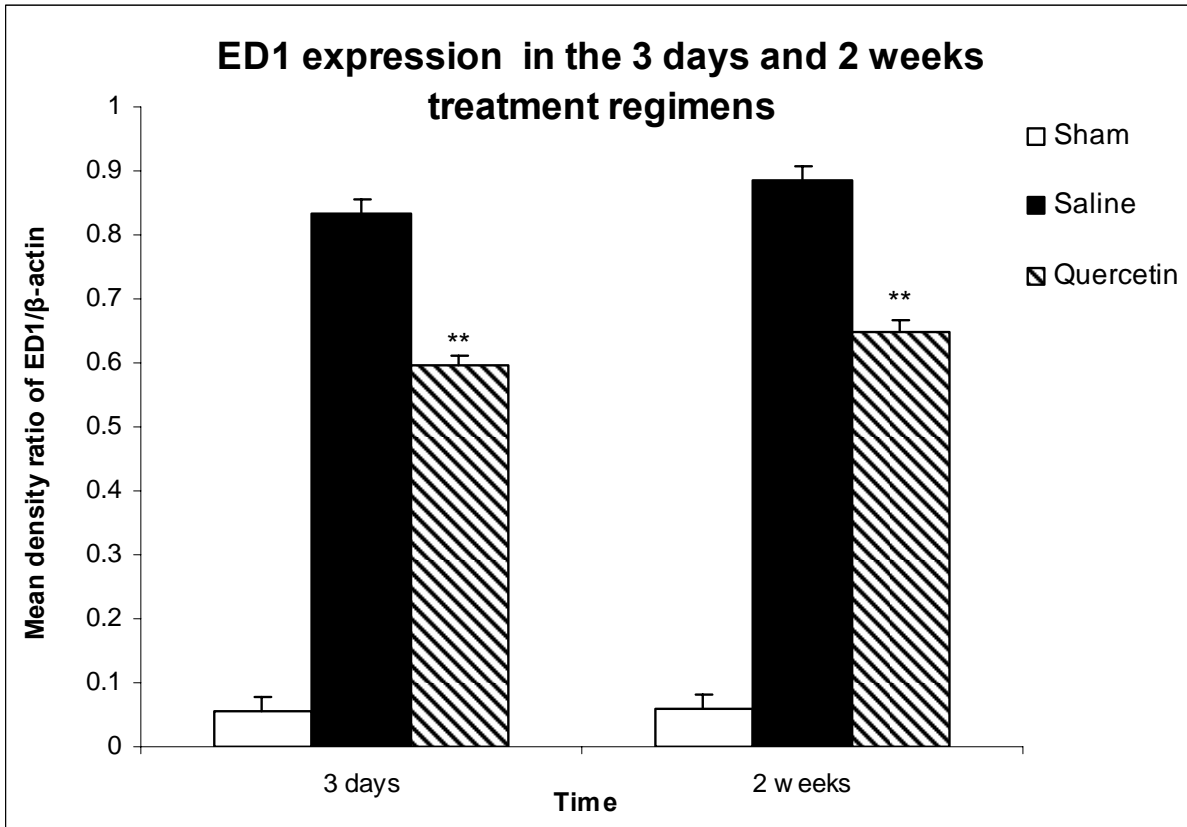


Fig 7.6b

FIGURE 7.6 (a) (b): Representative ED1 Western blotting (a) sham, saline and quercetin treated, from spinal cords harvested at eight weeks post surgery. Increased expression of ED1 is observed in all injured (saline controls and quercetin treated) compared to the sham animals, but is significantly greater in the saline as compared to quercetin treated. A plot (b) of the intensity of the bands from Western blots in two treatment regimens (i.e. 3 days and 2 weeks) show a statistically significant difference between saline and quercetin treated. Values are expressed as mean density ratio \pm standard error. ** denotes statistically significant difference between quercetin treated and saline control at $p < 0.001$. $n=6$ animals per group for each treatment regimen.

7.2.3 OX-42 expression

OX-42 is a marker for microglia and macrophages. Both saline-treated (n=6 per treatment regimen) and quercetin treated (n=6 per treatment regimen) animals had greater number of cells expressing OX-42 than the sham controls (n=6 per treatment regimen); however, there were fewer OX-42 positive cells in the quercetin treated animals compared to the saline control animals (Fig. 7.7). Western blotting analysis also showed similar findings (Fig. 7.8a). Tissues were analyzed from two treatment regimens (i.e. 3 days and 2 weeks quercetin treatment).

Analysis of the intensity of the bands from scanned Western blots showed a statistically significant increase of OX-42 expression in the saline control (n=6 per treatment regimen) compared to the quercetin treated animals (n=6 per treatment regimen) (Fig 7.8b). No significant difference was found between quercetin treated animals in various treatment regimens; indicating that the duration of treatment had no statistically significant effect on the treatment outcome.

7.3 MAPK activation (i.e. phosphorylation)

Phosphorylation of MAPKs usually indicates activation. The following MAPKs were examined: p38, JNK and ERK. The immunocytochemistry and western blot analyses showed a considerable increase in the activated (phosphorylated) state of these MAP kinases in the injured animal (i.e. all saline controls and quercetin treated animals) compared to the sham animals; however, the quercetin treated animals showed a significantly reduced activation of MAPKs compared to the saline control animals; indicating that quercetin treatment attenuated the activation of these kinases.

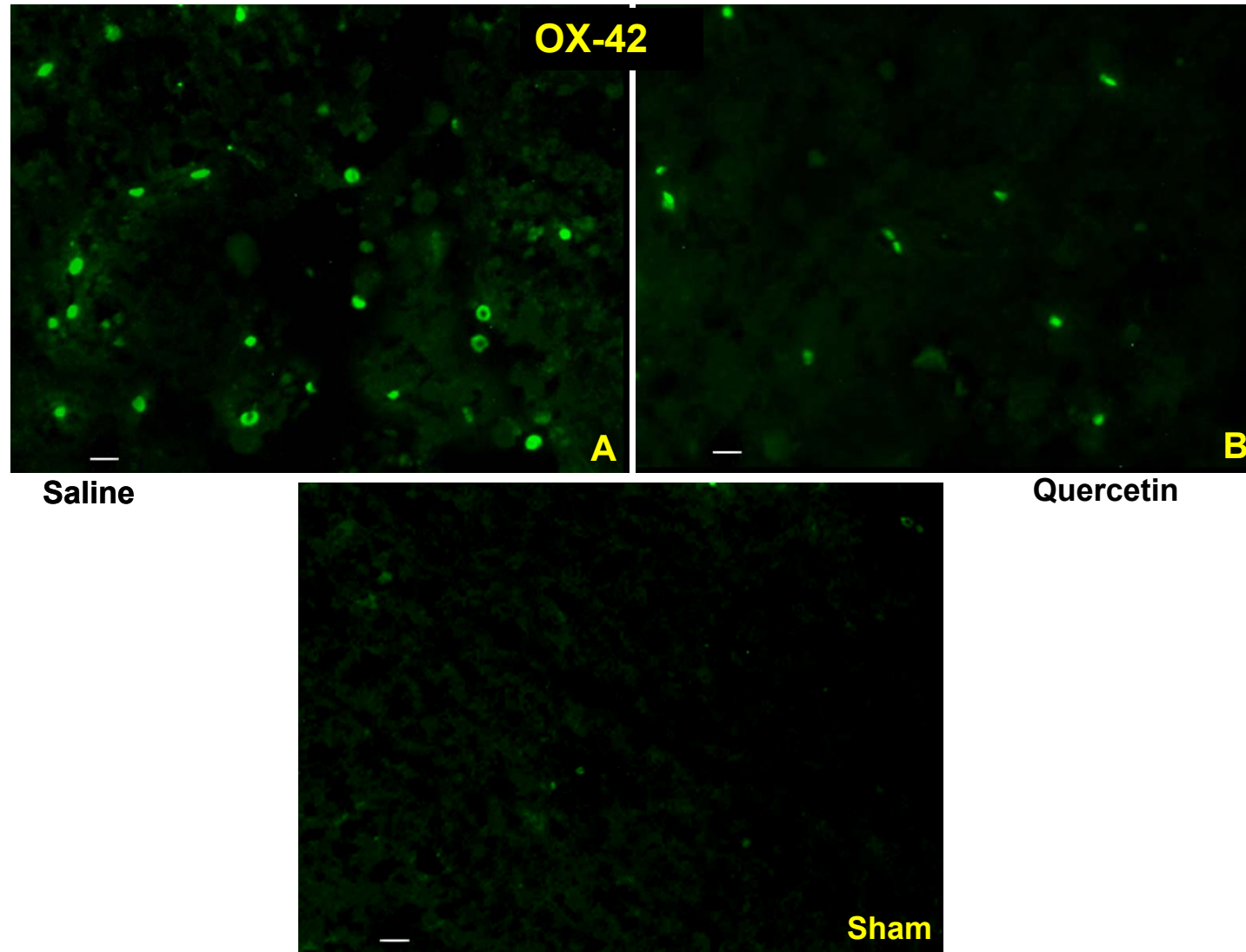


FIGURE 7.7: Representative photomicrograph of OX-42 immuno-staining in saline control A, quercetin treated B, and sham operated, using sections from spinal cords harvested at eight weeks post surgery. Saline control sections show more intense OX-42 signal compared to quercetin treated indicative of more activated macrophage/microglia in saline compared to quercetin treated. Sham animals show minimal signal. Scale bar = 50 μ m

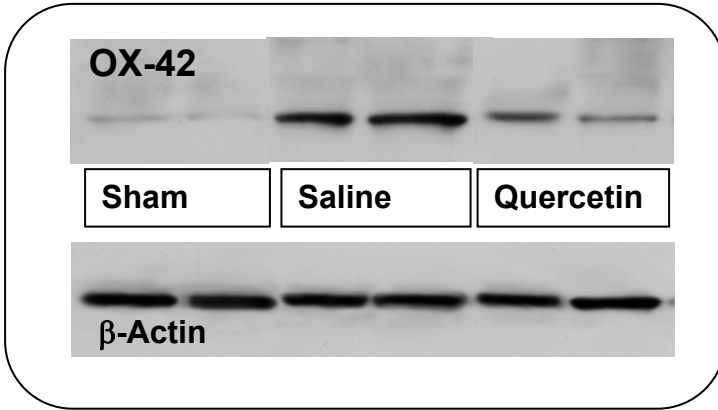


Fig 7.8a

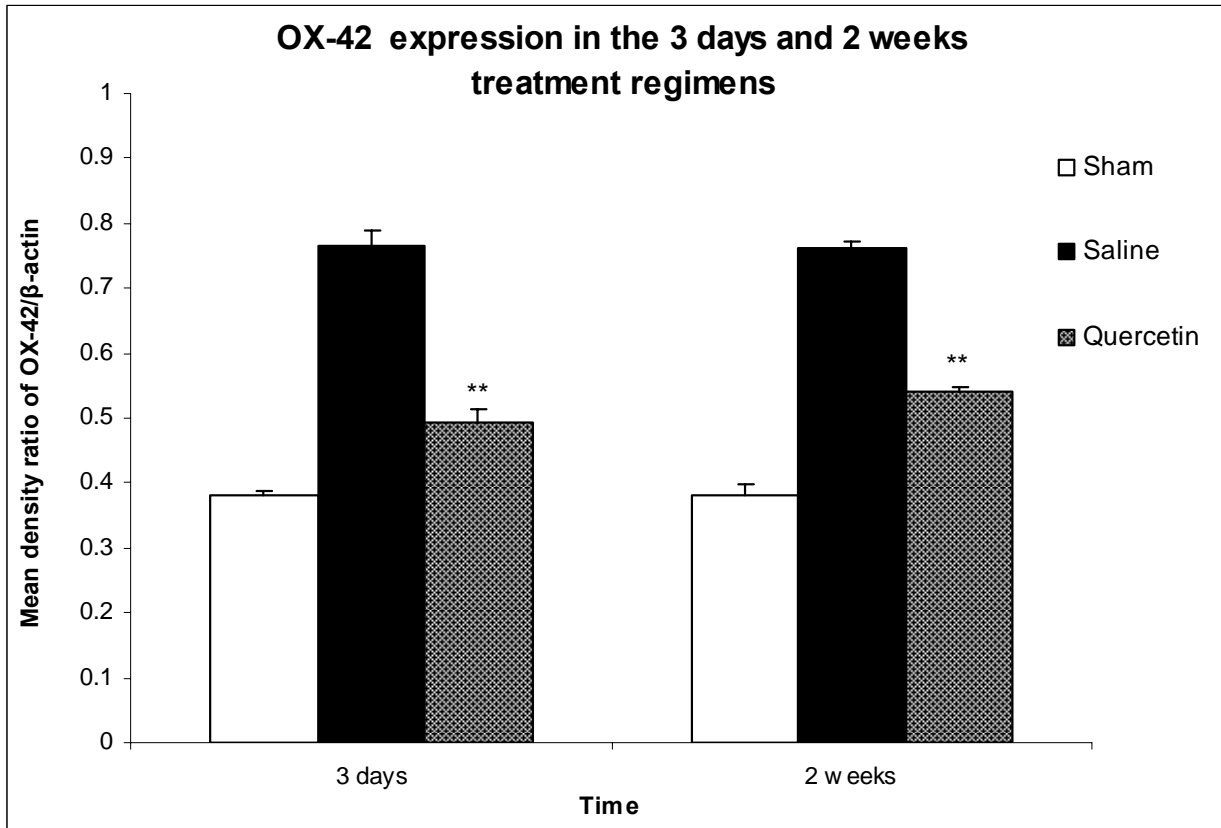
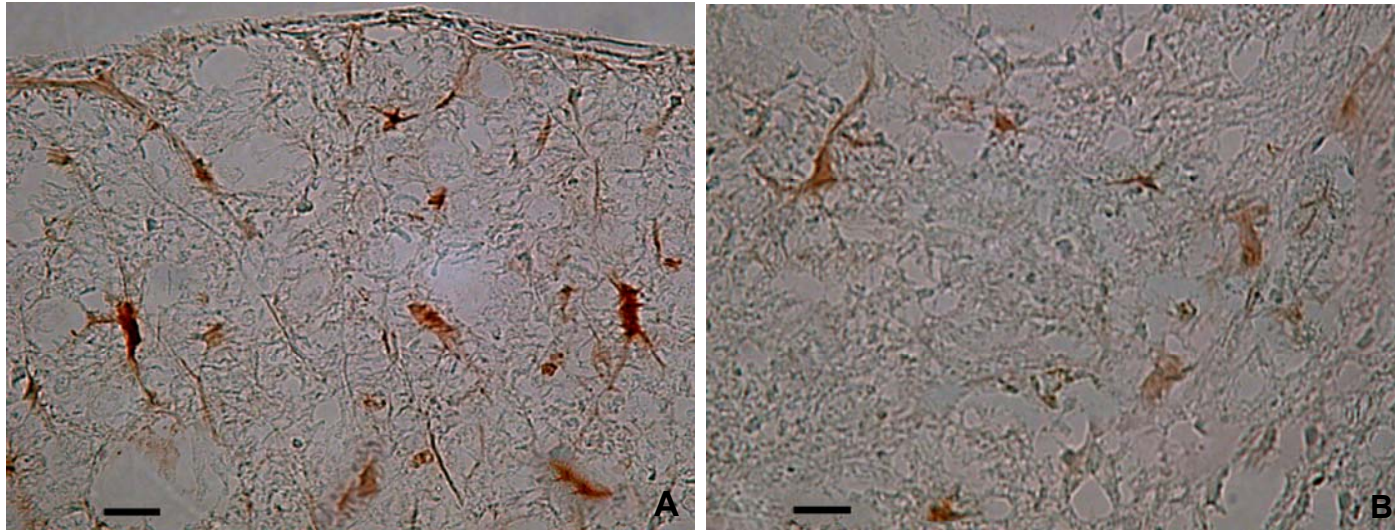


Fig 7.8b

FIGURE 7.8: Representative OX-42 Western blotting (a) from spinal cord tissue harvested at eight weeks post surgery from sham, saline control, and quercetin treated animals. Increased expression of OX-42 is observed in all injured animals (saline controls and quercetin treated) but show a significantly greater increase in the saline controls compared to quercetin treated. Analysis of the intensity of the bands from Western blots (b) in two treatment regimens (i.e. 3 days and 2 weeks) showed statistically significant higher increase in OX-42 expression in saline compared to quercetin treated. Values are expressed as mean density ratio \pm standard error. ** denotes statistically significant difference between quercetin treated and saline control at $p < 0.001$. $n = 6$ animals per group for each treatment regimen.

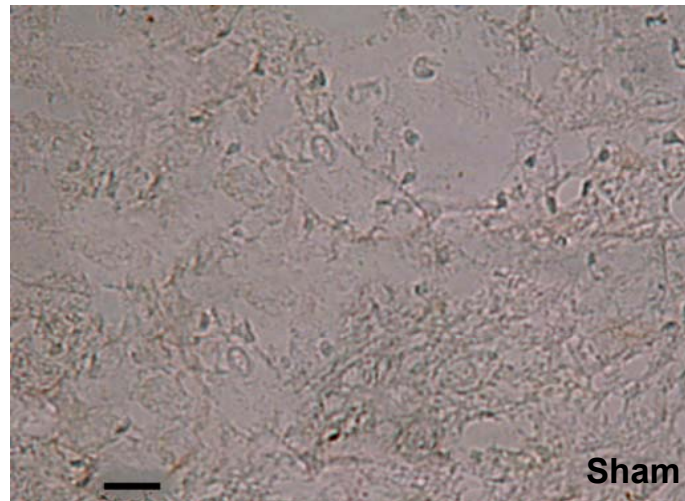
7.3.1 Phospho-MAPK p38:

Immunocytochemical staining for phospho-MAPK p38 from tissues harvested at 8 weeks post surgery from all treatment regimens (i.e. 3 days, 1 week and 2 weeks treatment) visually revealed increased activation of MAP kinase p38 in injured animals (saline controls and quercetin treated) compared to the sham animals (n=6 per treatment regimen) but there was more pronounced activation (phosphorylation) in the saline controls (n=6 per treatment regimen) compared to the quercetin treated (n=6 per treatment regimen) (Fig. 7.9). Phospho-MAPK p38 was present in stellate cells that morphologically appear to resemble astrocytes. An increased ratio of phospho-MAPK p38 to total MAPK p38 was seen in the Western blot analyses from both saline controls (n=6 per treatment regimen) and quercetin treated (n=6 per treatment regimen) animals (Fig. 7.10); indicating an increased activation state of this kinase in the injured animals compared to the sham animals. The expression of activated MAPK p38 was however more intense in the saline control animals compared to the quercetin treated animals. Comparison of the values obtained from quantification of the bands from Western blots showed a statistically significant higher increase in phospho-MAP kinases activity in the saline control compared to the quercetin treated animals in all treatment regimens; indicating that quercetin treatment attenuated the activation of these kinases. No statistically significant difference was found between the quercetin treated animals in various treatment regimens; indicating that the duration of treatment had no statistically significant effect on the treatment outcome.



Saline

Quercetin



Sham

FIGURE 7.9: Representative photomicrograph of phospho-MAPK p38 immuno-staining in saline control A, quercetin treated B, and sham operated, using spinal cord sections for from tissue harvested at eight weeks post surgery. Saline control sections display more cells with astrocytes-like morphology showing more intense phospho-MAKP p38 signal compared to quercetin treated; indicative of more activated MAPK p38 in the saline controls compared to quercetin treated. Scale bar = 50 μ m.

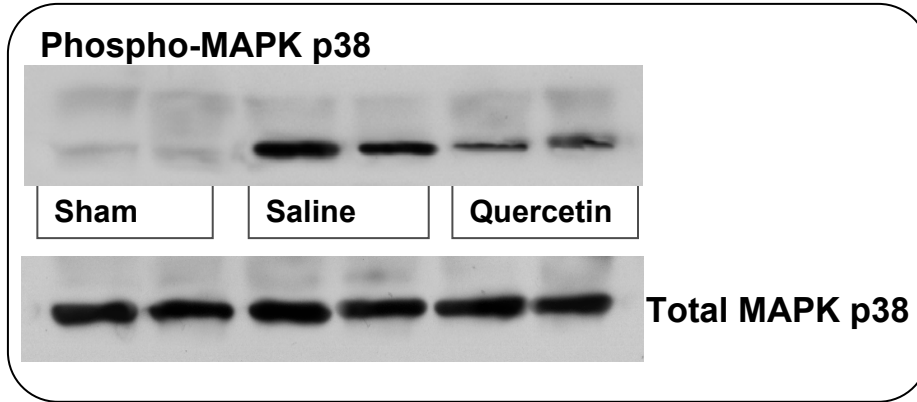


Fig 7.10a

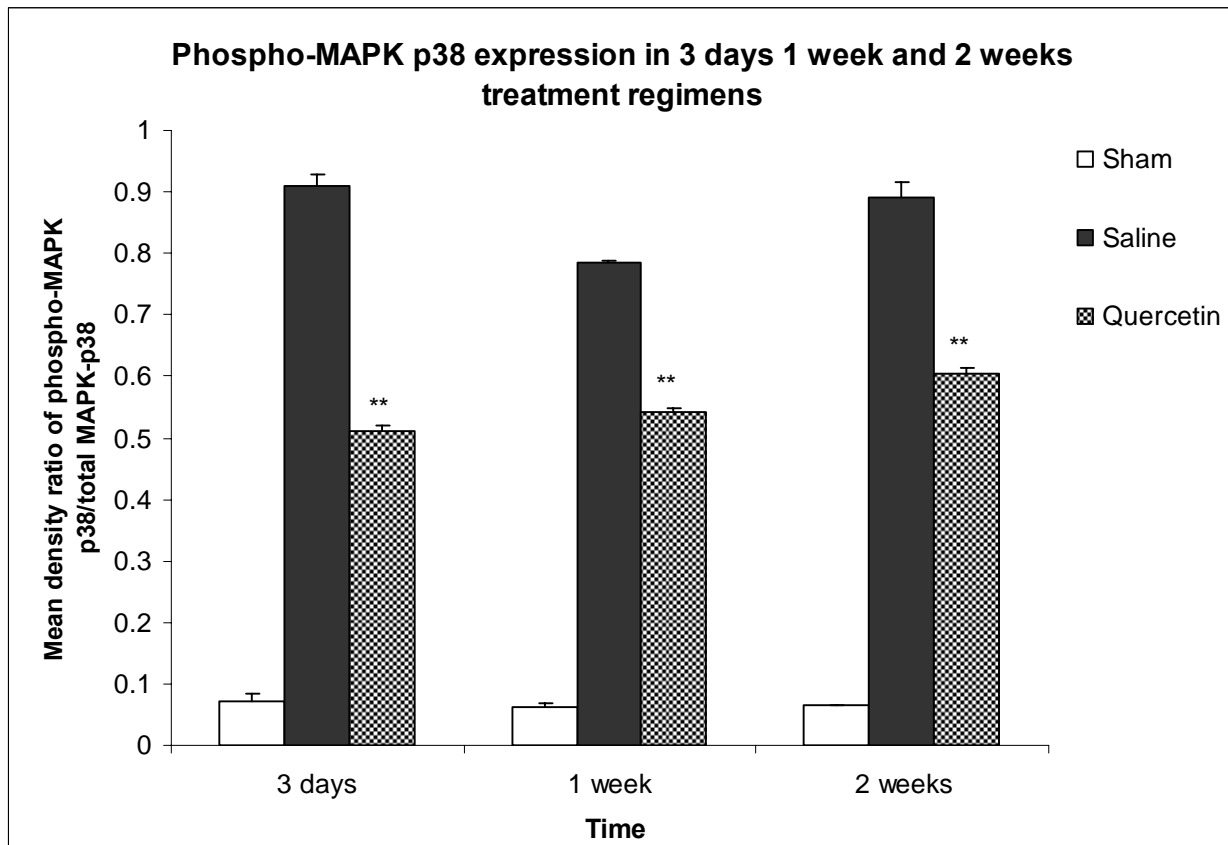


Fig 7.10b

FIGURE 7.10 (a) (b): Representative phospho-MAPK p38 Western blotting (a) in sham, saline control and quercetin treated from spinal cords harvested at eight weeks post surgery. Increased level of phospho-MAPK p38 is observed in both saline control (n=6) and quercetin treated (n=6) compared to the sham animals (n=6); nevertheless, MAPK p38 phosphorylation is significantly higher in the saline controls compared to the quercetin treated animals in all treatment regimens (i.e. 3days, 1 week and 2 weeks). A graph of the analysis of the intensity of bands (b) from Western blots in all treatment regimens shows significantly higher phospho-p38 levels in saline controls compared to the quercetin treated. Values are expressed as mean density ratio \pm standard error. ** denotes statistically significant difference between quercetin treated and saline control at $p < 0.001$. n=6 animals per group for each treatment regimen.

7.3.3 Phospho-SAPK/JNK:

Similar to the MAPK p38, immunocytochemistry for phospho-SAPK/JNK visually revealed increased activated (phosphorylated) SAPK/JNK in the spinal cord injured animal (i.e. saline controls and quercetin treated) compared to the sham animals (n=6 per treatment regimen). However, sections from the saline control animals (n=6 per treatment regimen) displayed more cells with activated SAPK/JNK as well as more intense signal compared to the quercetin treated (n=6 per treatment regimen). Similar to the phospho-MAPK p38, a lot of these positive cells for activated MAPK SAPK/JNK presented stellate morphology (Fig. 7.13.). Western blotting analysis showed an increased ratio of phospho-SAPK/JNK to total SAPK/JNK (Fig 7.14) in spinal injured animals (i.e. saline controls and quercetin treated) compared to the sham animals (n=6 per treatment regimen); again indicating an increased activation state of SAPK/JNK in the injured animals compared to the sham animals. Also, expression of activated MAPK p38 was significantly more intense in the saline control animals (n=6 per treatment regimen) compared to the quercetin treated animals (n=6 per treatment regimen). A plot of the values obtained from bands from various scanned Western blots is shown in Fig. 7.11. Comparison of the values of Western blot quantification with ANOVA showed a statistically significant lower ratio of phospho-SAPK/JNK/total SAPK/JNK in the quercetin treated animals (n=6 per treatment regimen) compared to saline control animals (n=6 per treatment regimen); indicating that quercetin treatment attenuated the activation of the kinase. However, there was no statistically significant difference between the quercetin treated in various treatment schedules; indicating that the duration of treatment had no statistically significant effect on the treatment outcome. Tissues were analyzed from all three treatment regimens (i.e. 3 days, 1 week and 2 weeks quercetin treatment).

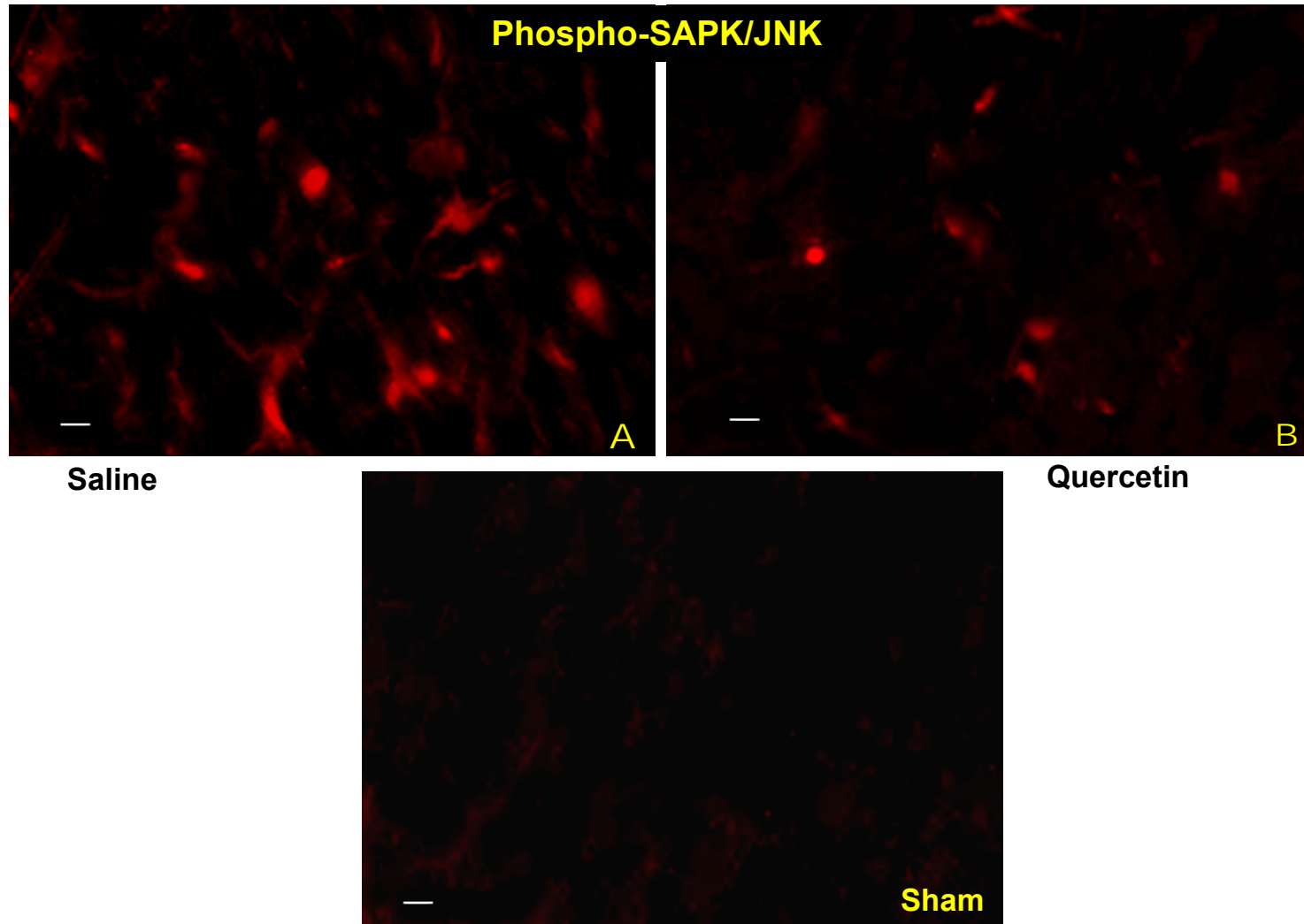


FIGURE 7.11: Representative photomicrograph phospho-SAPK/JNK immunoreactivity in saline control A, quercetin treated B, and sham operated, using sections from spinal cord tissue harvested at eight weeks post surgery. More positive cells (a lot of which display stellate morphology) with more intense signals are present in the saline control compared to the quercetin treated sections. Sections from sham animals show minimal immunoreactivity. Scale bar = 50 μ m

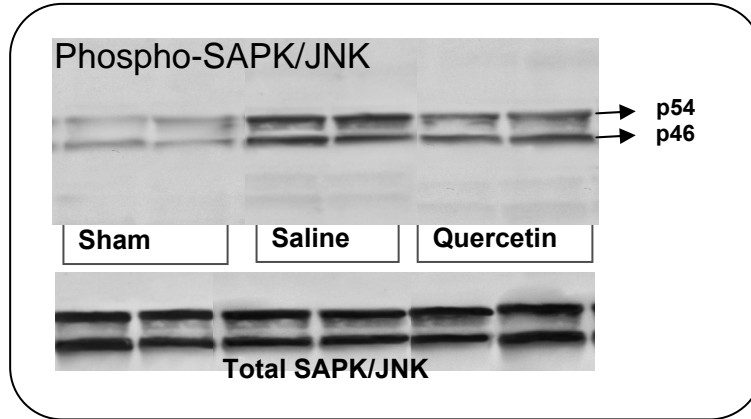


Fig 7.12a

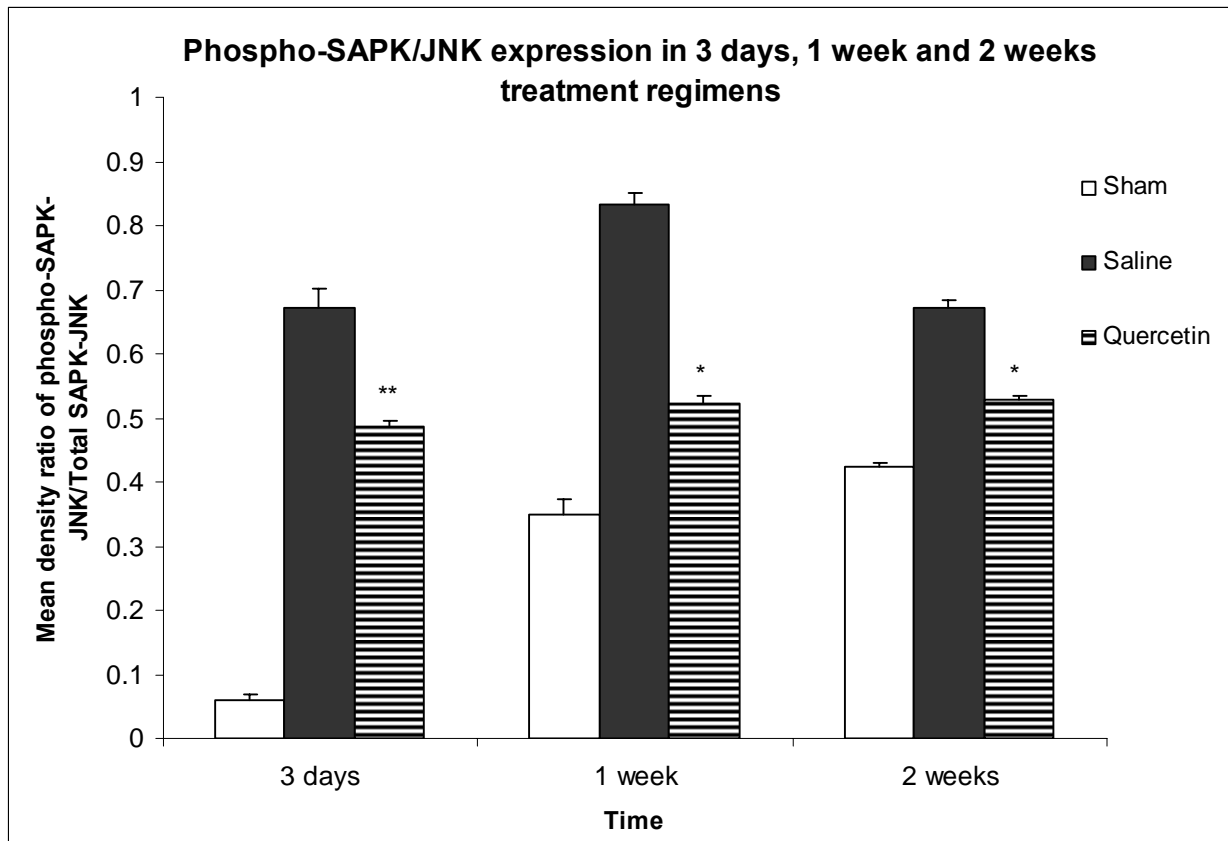


Fig. 7.12b

FIGURE 7.12: (a) Representative phospho-SAPK/JNK Western blotting in sham, saline control and quercetin treated animals from spinal cords at harvested at eight weeks post surgery. There is an increased expression of phospho-SAPK/JNK in all injured animals (i.e. saline control and quercetin treated) compared to the sham animals; however, phospho-SAPK/JNK expression is significantly higher in the saline control (n=6) compared to the quercetin treated animals (n=6) in all treatment regimens (i.e. 3 days, 1 week and 2 weeks). A graph of the analysis of the intensity of bands (b) from Western blots in all treatment regimens, shows significantly increased expression of activated SAPK/JNK in the saline controls compared to the quercetin treated animals. Values are expressed as mean density ratio \pm standard error. * and ** denotes statistically significant difference between quercetin treated and saline control at $p < 0.01$ and $p < 0.001$. n=6 animals per group for each treatment regimen.

7.3.2 Phospho-MAPK ERK 1/2 (p 44/42):

Visual examination of slides immuno-stained for phospho-ERK 1/2, showed increased positive cells in both saline control and quercetin treated compared to the sham operated animals (n=6 per treatment regimen) but the quercetin treated (n=6 per treatment regimen) showed reduced ERK 1/2 activation compared to the saline control (n=6 per treatment regimen) animals (Fig. 7.13). Many the positive cells also reveal stellate morphology. Western blotting analysis confirmed increased activated state of phospho-ERK 1/2 in all injured spinal animals (saline controls and quercetin treated) compared to the sham animals with the quercetin treated (n=6 per treatment regimen) showing a reduced activation compared to the saline control (n=6 per treatment regimen) animals (Fig 7.14). Comparison of the values obtained quantification of the bands from various scanned Western blots showed a statistically significant lower phospho-ERK 1/2/total ERK 1/2 ratio in the quercetin treated (n=6 per treatment regimen) compared to the saline control (n=6 per treatment regimen) animals; indicating that quercetin treatment attenuated the kinase activation. There was also no statistically significant difference between the quercetin treated in the different treatment schedules; indicating that the duration of treatment had no statistically significant effect on the treatment outcome. Tissues were analyzed from all three treatment regimens (i.e. 3 days, 1 week and 2 weeks quercetin treatment).

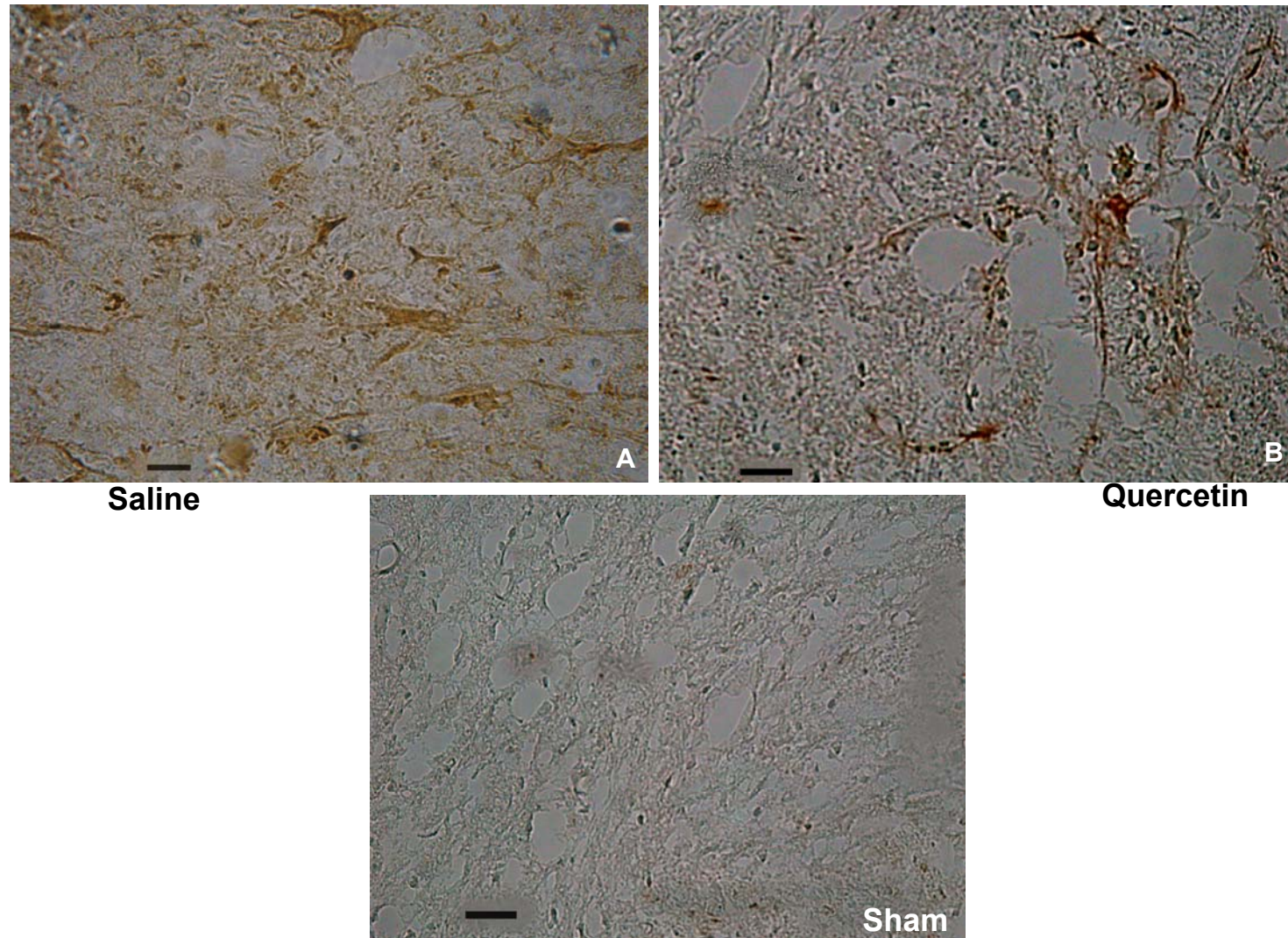


FIGURE 7.13: Representative photomicrograph of phospho-ERK1/2 immunostaining in saline control A, quercetin treated B, and sham operated, using sections from spinal cord tissue harvested at eight weeks post surgery. Saline control sections show more intense signal (with a lot of the cells displaying stellate morphology) compared to quercetin treated; indicative of more activated ERK 1/2 in the saline controls compared to the quercetin treated. Sham sections show minimal signal. Scale bar = 50 μ m

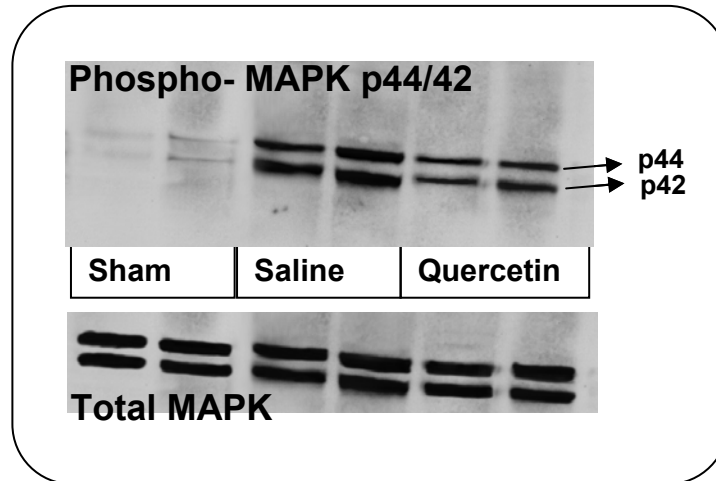


Fig. 7.14a

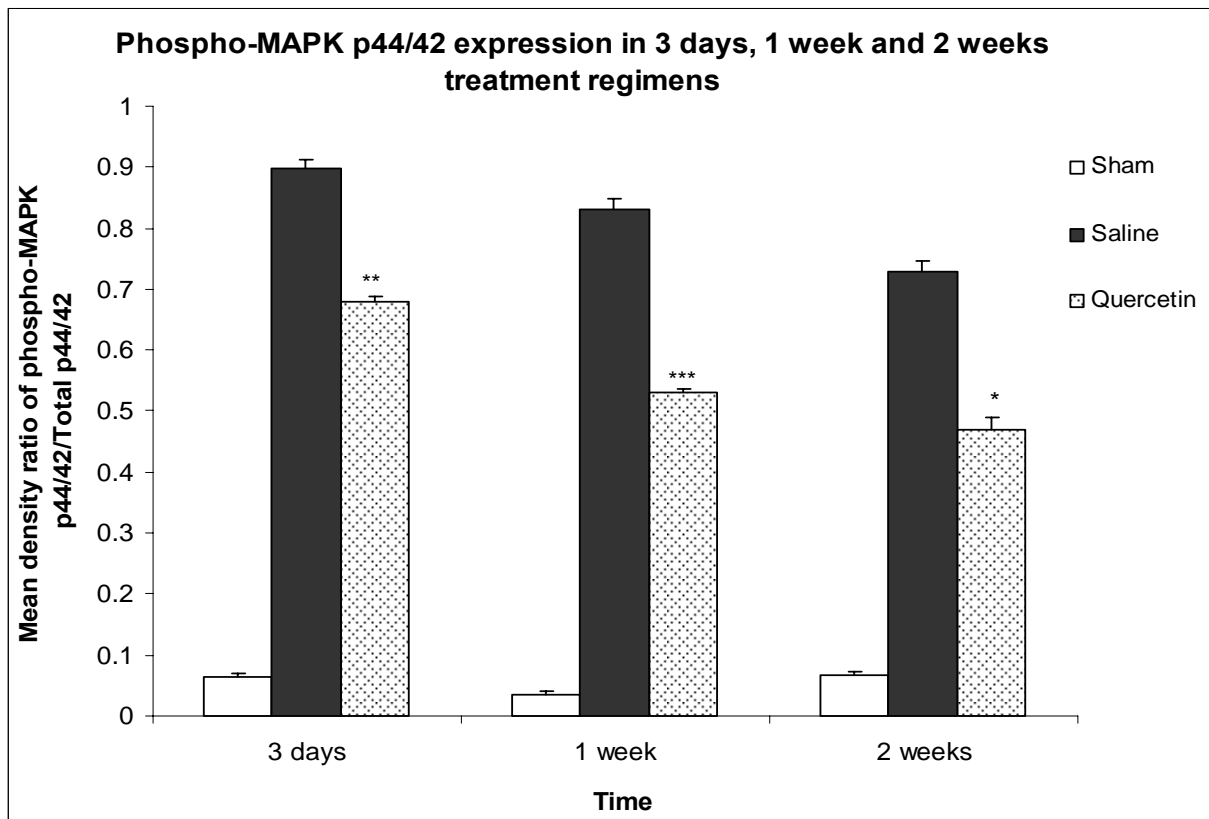


Fig. 7.14 (b)

FIGURE 7.14: (a): Representative phospho-ERK 1/2 (p44/42) Western blotting in sham, saline control and quercetin treated animals from spinal cords harvested at eight weeks post surgery. Increased expression of phospho MAPK p44/42 is observed in all injured animals (i.e. saline control and quercetin treated) compared to the sham animals (n=6); however, the expression of activated MAPK 44/42 is significantly higher in saline controls (n=6) compared to quercetin treated (n=6) in all treatment regimens. A graph of the analysis of the intensity of bands (b) from Western blots shows significantly higher increases in the saline controls compared to quercetin treated animals in all treatment regimens. Values are expressed as mean density ratio \pm standard error. * denotes statistically significant difference between quercetin treated and saline control at $p < 0.01$. ** and *** denotes statistical significance at $p < 0.001$ and 0.0001 . n=6 per group per each treatment regimen.

CHAPTER 8.0

GENERAL DISCUSSION, FUTURE DIRECTIONS AND CONCLUSIONS

8.1 Introduction

Quercetin, one of the most abundant flavonoids in the human diet, has been reported to exhibit a wide range of pharmacological properties, including anti-oxidant, anti-allergic, anti-inflammatory and anti-apoptotic effects (Huk, 1992; Cotelle, 1996; Siaji, 1995; Ferrandiz, 1991; Middleton and Kandaswami, 1992; Middleton et al., 2000; 1992; Pelzer, 1998; Lamson et al., 2000; Rahman et al., 2006; Mullen et al., 2006; Graf et al., 2006; Moon et al 2008). The neuroprotective actions of quercetin have been reported in various studies (Schültke et al 2003 and 2010; Ossola et al., 2008, 2009; Bureau et al., 2008; Ansari et al., 2009). The inherent complexity of the biological system coupled with the many potential beneficial actions of quercetin make it difficult to determine with specificity each of the mechanisms by which quercetin produces its effects. Nevertheless, in this study, the neuroprotective effects of quercetin were observed already by the first week after treatment (3 weeks post SCI surgery) in all treatment regimens (as evidenced by the results from behaviour studies). It is known that within this time frame after traumatic injury to the spinal cord, glia scar formation and apoptosis are prominent (Beattie et al., 2000; Fawcett and Asher, 1999; Crowe et al., 1997; Emery et al., 1998) raising the possibility that signaling pathways that lead to these processes were important targets of this drug.

Previous studies in our lab have reported that quercetin administration in acute SCI is associated with decreased activation of systems that promote oxidative stress and inflammatory responses (such as myeloperoxidase activity, reduced iron clearance) after acute SCI (Schultke et

al., 2003 and 2010). Since these systems are also still active during chronic insult, it is possible that quercetin's actions in chronic spinal cord injury also involve attenuation of these mechanisms.

The data shown in chapters 5, 6 and 7 demonstrate the ability of quercetin to act as a neuroprotective agent in traumatic SCI. Traumatic SCI produces tissue damage that continues to evolve days and weeks after the initial insult, with corresponding functional impairments. Modern management following mechanical trauma often requires operative treatment for decompression of the cord and stabilization of the spine structure. In spite of surgical treatment, neurodegenerative processes of the nervous tissue still progress. Reducing the extent of progressive tissue loss should result in a better recovery from SCI, but drug treatment options have thus far been limited. Methylprednisolone (MPO) is one of the very few drugs widely used in the management of acute traumatic SCI but its application still remains a very controversial topic (Botelho et al., 2009; Walsh et al., 2010; Bracken et al., 1990) as this therapy carries a substantial risk of adverse side effects.. Recently, numerous studies have demonstrated several neurodegenerative pathways that occur after the direct damage of nervous tissue including signaling mechanisms that are involved in the chronic stages of this condition (Lu et al., 2010; Duan et al., 2010).

In this study, we demonstrated the effect of delayed quercetin treatment on locomotor functional recovery following chronic experimental SCI in adult male Wistar rats. We also demonstrated the effects of quercetin on axon function, inflammatory processes and apoptosis as well as its influence the regulation of MAP kinase activity.

8.2 The effect of delayed quercetin administration on locomotor functional recovery and tissue preservation following spinal cord injury

8.2.1 Behaviour studies

In this study, motor functions of the hind-limb were evaluated by the BBB locomotor scale (Basso et al., 1995) and by the modified incline plane scale (Rivlin and Tator 1977), showed better locomotor outcome in all the quercetin treated animals (n=18 i.e. 6 animals per each of the three treatment regimens) (Fig. 5.1 and 5.4). In the absence of quercetin intervention not one of the spinal cord injured saline control animals (n=18 i.e. 6 animals per each of the three treatment regimens) attained a BBB score beyond 5 points whereas all of the delayed quercetin treated animals (n=18 i.e. 6 animals per each of the three treatment regimens) had BBB scores of 8 or above with the highest scores reaching 11 points (i.e. between 8-11 points).

The model of spinal cord injury that we used (compression injury) is clinically relevant since the majority of patients with traumatic SCI have incomplete injury. After inducing SCI, both reflexes and voluntary motor functions below the level of the injury are initially lost; partial recovery may occur over time (Basso et al., 1995; Ko et al., 1999; Gale et al., 1985). The recovery of functions mediated by supraspinally controlled reflexes is slow and incomplete given that they require the function of long tracts, many of which are irreversibly damaged by the injury (Hiersemenzel et al., 2000; Leis et al., 1996). Recovery of locomotion and limb placement depends on ascending and descending spinal cord tracts, including cortico-, rubro-, reticulo-, vestibulo-, and raphe-spinal tracts (Basso, 2000). The anterolateral and posterior funiculi are known to contain tracts responsible for hind-limb function (Iizuka et al., 1997). The functional loss after SCI in rats involves interruption of descending serotonergic, reticulospinal, and other

descending spinal tracts that facilitate segmental reflexes (Saruhashi et al., 1994; Gruner et al., 1996). The ascending spinothalamic tracts mediate the perception of pain and temperature below the level of the lesion (not explored in this study). Since locomotor functional recovery was better in quercetin treated animals than in the saline control animals, it appears that quercetin preserved the function of multiple long tracts.

8.2.2 Histology studies

Results from histology revealed that improvement of locomotor functional recovery was associated with increased neural tissue survival and myelin preservation. These analyses showed less cell damage, lesser cavitations and better preservation of tissue structure in quercetin treated (n=18 i.e. 6 animals per each of the three treatment regimen) compared to the saline controls (n=18) (Fig. 5.6). Quantification of white matter content evidenced with LFB staining (Fig. 5.7) showed significantly more white matter content (including the anterolateral and posterior funiculi) in the quercetin treated animals compared to the saline controls indicating that better myelination was due to quercetin application. It is also known that axonal durability after SCI depends on the presence of myelin. Therefore quercetin may be protecting axons through its ability to influence myelination.

The current study did not analyze data on the effect of quercetin on the time of return to normal bladder function. This would be an interesting aspect to explore in future studies. Normal micturition requires coordinated activation of the bladder's smooth muscle (detrusor) and the striated muscle of the external urethral sphincter, controlled by spinal and supraspinal circuitry (de Groat, 1990). Thus after SCI, initially bladder function is lost, but it is later partially recovered; the extent of recovery depending on the degree of preservation of white matter (and

hence the long tracts) at the injury site (Pikov and Wrathal 2001; Pikov et al., 1998). Since our work has shown that locomotor recovery was associated with white matter preservation it could be postulated that quercetin application would be associated with a reduction of the time of return to normal micturition in spinal cord injured animals.

8.3 The effects of quercetin on axon function and inflammatory responses

Our study also aimed to reduce the extent of inflammation and glial scar formation in injured spinal cord using quercetin. Results showing the effects of quercetin on axon function and inflammatory processes, including reactive gliosis, the putative factor in tissue scarring are reported in chapter 7. In both saline control and quercetin treated animals, positive immunoreactivity using ED1, OX42 and GFAP antibody were observed around the injured site indicating that inflammatory responses were still present at 8 week after the injury. It has been reported that inflammation after SCI diminishes by 2 weeks post injury (Li et al., 1999; Horiuchi et al., 2003). Even though these reports are factual, our findings suggest that the remaining inflammatory responses are effective enough to contribute to the negative progression of the disease. β -APP accumulation (indicative of axoplasmic transport impairment) was much more pronounced in the saline control animals (n=6 per treatment regimen) compared to those that were treated with quercetin (n=6 per treatment regimen) (Fig. 7.1 and 7.2) indicating that quercetin positively influences axon function by improving axoplasmic transport. Similarly, we found a more pronounced ED1, OX-42 and GFAP expression (from immunocytochemistry and Western blot analysis) in the saline control animals (n=6 per treatment regimen) compared to the quercetin treated (n=6 per treatment regimen) (Fig. 7.3, 7.4.7.5, 7.6, 7.7 and 7.8) indicating that quercetin contributed in reducing the inflammatory response following SCI. In addition, GFAP

expression is typical of reactive gliosis, another component of late inflammatory and immunological responses that leads to the formation of glia scar. These results suggest that quercetin maybe useful in attenuating the formation of glial scar. Reports from various studies have linked the process of reactive gliosis with increased MAP kinase activity (Chiu et al., 2010; Zhuang et al 2007; Watts et al., 2010; Matsushita et al., 2008; Yamasaki et al., 2007; Schieven, G. L, 2005; Zarubin and Han, 2005; Schieven, 2005; Zarubin and Han, 2005). The central role of MAPKs in inflammation and cell death has been widely established (Watts et al., 2010; Matsushita et al., 2008). MAPKs appear to be major contributors to secondary damage in trauma that involves the CNS as well as in other neurodegenerative diseases (Raoul et al., 2006; Horiuchi et al., 2003).

8.3.1 Axon function

Axonal pathology following SCI is profuse (Blight, 1988; Gentleman et al., 1995; Maxwell et al., 1997) hence the preservation of functional axonal connections after trauma is an essential goal of any therapeutic intervention. Diffuse axonal damage appears to be responsible for a considerable amount of the post-traumatic CNS dysfunction (Gentleman et al 1995; Maxwell et al., 1997) and evidence suggests that much of this damage is secondary to impairment in axoplasmic transport (Povlishock, J.T., et al., 1999). Interference with axoplasmic transport is known to cause diffuse accumulation of β -APP within neuronal perikaryon (Van Den Heuvel et al., 1998) which can be detected by examining for accumulation of β -amyloid precursor protein (β -APP) (Gentleman et al 1995).

There are a number of factors involved that may result in axoplasmic transport impairment (see section 1.5.2) including elevated intracellular Ca^{2+} and production of free

radicals such as ROS when cells are subject to oxidative stress (Povlishock et al., 1999; Juurlink and Paterson, 1998; Dawson et al., 1993; Tanaka et al., 1994). The release of ROS during secondary insults damages cellular lipids, proteins and nucleic acids. In addition, there are immunological changes that result in increased production of cytotoxic inflammatory mediators further contributing to neuronal and glial injury (Carlos, T.M., et al., 1997; Giulian et al., 1994; McKeating et al., 1998; Fawcett and Asher, 1999; Mazzanti et al., 2001; Norenberg, 1994, 1996). It can therefore be understood that these mechanisms can be ameliorated if neuronal anti-oxidant defences are increased (Su and Murphy, 1999). Since quercetin is known to possess very strong anti-oxidant properties, and its administration reduced β -APP accumulation in spinal cord injured animals, this could be one mechanism by which it improves axon function. This could have implications in other neurodegenerative diseases such as Alzheimer's disease (AD) that is characterized by β -APP aggregation and neuritic plaque formation.

8.3.2 Inflammatory responses

Inflammatory responses are known to play a crucial role in the series of rapid pathophysiological changes that are believed to be triggers for the subsequent damage that lead to neurodegeneration after traumatic damage to the CNS. These responses, are typically detected histologically as expression of cell markers such as ED1 and OX-42 (Fawcett et al., 1999; Westmoreland et al., 1996; Carlson et al., 1998; Chao et al., 1992; Schubert et al., 1998; Viviani et al., 1998). Our results show that administration of quercetin is associated with a significant decrease in ED1 and OX-42 (activated macrophages/microglia) positive cells present in the spinal cords of the quercetin treated animals (n=6 per treatment regimen) compared to the saline controls (n=6 per treatment regimen) confirming the anti-inflammatory properties of quercetin.

Increased immunoreactivity for glial fibrillary acidic protein (GFAP) is a typical characteristic of reactive gliosis; a combination of glia responses, which eventually lead to the formation of a glial scar and inhibit axonal regeneration (Fawcett and Asher, 1999). The mechanism of the scar formation is complicated, since many glial cells such as microglia, oligodendrocyte precursors, meningeal cells, and astrocytes proliferate and/or migrate to the injured site to form the glial scar (Ridet et al., 1997; Fawcett and Asher, 1999). Moreover, numerous related cytokines and growth factors participate in glial cell proliferation and/or differentiation (Ridet et al., 1997). The evidence also implicates MAP kinase (including p38 and ERK 1/2 and JNK) signaling pathways (Zhang et al., 1998; Bhat et al., 1998) in these responses. Therefore, a combination of two or three methods may be necessary to attenuate glial scar formation. Quercetin could be affecting one or more of these pathways.

8.4 The effects of quercetin on apoptosis and MAP kinase activity in chronic spinal cord injury

TUNEL analyses done on spinal cord tissue harvested at 8 weeks post injury showed a significant reduction of positive cells in the quercetin treated (n=6) compared to the saline control animals (n=6 per treatment regimen) in all three treatment schedules (results reported in chapter 6, Fig. 6.1 and 6.2). These results were endorsed by results from immunocytochemistry and Western blot analysis for activated caspase 3 and cleaved PARP (Fig. 6.3, 6.4, 6.5 and 6.6).

Amongst the intracellular pathways that have been identified to mediate delayed neuroinflammatory responses (such as reactive gliosis) and apoptosis are the mitogen-activated protein kinase (MAPK) families. Activation of MAPK p38, JNK and ERK has been widely implicated in neuronal and glia apoptosis. We found that the activation of these three kinases

(detected by immunocytochemistry and western blot analysis) was significantly more pronounced in the saline control animals (n=6 per treatment regimen) compared to the quercetin treated animals (n=6 per treatment regimen) (Fig. 7.9, 7.10, 7.11, 7.12 7.13 and 7.14). These results indicate that the protective effects of quercetin on reactive gliosis and apoptosis could be linked to its suppressive effect on MAP kinase activation.

8.4.1 Apoptosis

The anti-apoptotic effects of quercetin have been documented in various studies (Ishikawa et al., 2000; Borska et al., 2003; Chao et al., 2009) and this is another potential means through which quercetin might exert beneficial effects after spinal cord injury. The apoptotic process and its cellular responses are mediated through the expression of different sets of proteins and various cellular products involved in the execution of apoptotic and anti-apoptotic events (Kerr et al., 1972; Wyllie et al., 1980; Schuler and Green, 2001; Freidlander et al., 2003; Beattie et al., 2002b). Important key players in apoptosis are the cysteine dependent aspartate specific protease (caspases), and PARP (poly ADP-ribose polymerase). Caspases are expressed as latent zymogens and are activated by an autoproteolytic mechanism or by processing by other proteases (frequently other caspases). The executioner caspase, caspase-3 is said to be the merger of the two major biochemical pathways (extrinsic and intrinsic) of apoptosis. This caspase is characterized by a short N-terminal pro-domain and in the presence of various stress signals it is activated by an upstream caspase leading to transactivation of a number of downstream elements including phosphorylation of histones (such as H2AX at serine 149 and H2B at serine 14), cleavage of PARP, inter-nucleosomal degradation of DNA, and packaging of the cell into small units that are easily taken up by neighbouring cells (section 1.5.4.2). Hence, increased expression

of activated caspase 3 and proteolytic cleavage of PARP as well as degradation of DNA are important hallmarks of apoptosis that were exploited in this study.

It is recommended that discrimination of apoptosis be analyzed by more than one method since a single apoptosis assay may not work in all cell conditions. In the beginning of necrosis for example, nuclei may be TUNEL-positive since the DNA is being degraded by DNAses. Activation of poly(ADP-ribose) polymerase (PARP) by DNA breaks catalyzes poly(ADP-ribosylation) and results in depletion of NAD^+ and ATP, which is thought to induce necrosis. However, cleavage of PARP by caspase prevents induction of necrosis during apoptosis and ensures appropriate execution of caspase-mediated programmed cell death (Zdenko Herceg and Zhao-Qi Wang, 1999). Hence to ensure that possible errors of TUNEL method are minimized we complemented this method with immunocytochemical staining and Western blot analyses for activated Caspase 3 and cleaved PARP.

Apoptosis after SCI has been described by many investigators (Beattie et al., 2000; Beattie et al., 2002; Casha et al., 2002; McDonald and Belegu, 2006; Crowe et al., 1997). In these reports, early apoptosis of neural cells, including neurons, is followed by a delayed wave of predominantly apoptotic cell death in degenerating white matter tracts. Studies of apoptosis in white matter after injury raise the possibility that glial apoptosis occurs, at least in part, as a consequence of axonal degeneration (Abe et al., 1999; Warden et al., 2001). However, the presence of activated microglia in contact with apoptotic oligodendrocytes after SCI indicates that this interaction may also activate cell death programs in the oligodendrocyte (Shuman et al., 1997) which may then be followed by secondary axonal degeneration (Bjartmar et al., 1999; Yin et al., 1998). In this study, we found that quercetin significantly suppressed reactive gliosis as well as apoptosis in the injured spinal cords and this was associated with improved neurological

outcome. By indications from these studies, it seems reasonable to postulate that quercetin could be improving axon survival by inhibiting glia cell apoptosis. Therefore quercetin treatment at 2 weeks appear rescue glia cells thereby allowing survival of axons and supporting cells that normally would die somewhere between 2 and 6 weeks after injury.

8.4.2 MAP kinase activity

The effects of quercetin on activation (phosphorylation) of various mitogen-activated protein kinases (MAPK) that are known to be implicated in neurodegeneration (including inflammation, oxidative stress and apoptosis) after SCI are also shown in chapter 7. Our results show that quercetin afforded considerable neuroprotection against SCI induced cell death in parallel to its inhibition of p38, SAPK/JNK and p44/42 kinase activation. The mechanism of quercetin's inhibition of these kinase activation is not clear but it may some how interfere with inflammatory and apoptotic responses, that induce potential signals for MAP kinase activation. It is also known that quercetin is a non-specific inhibitor of MAP kinases (Graziani et al., 1981; Rubio et al., 2007; Chiu et al., 2010).

The MAPK families such as p38, SAPK/JNK and p44/42, have provided topics for discussion with reference to their roles in delayed neuronal damage as well as reactive gliosis and apoptosis (Chiu et al., 2010; Zhuang et al 2007; Schieven, 2005; Zarubin and Han, 2005; Widmann et al., 1999; Kyriakis and Avruch, 2001; Ip and Davis 1998; Christman et al., 2000; Baeuerle and Henkel, 1994; Wolf and Seger, 2002 Mattson et al., 2001; Takman et al., 2004; Nakahara et al., 1999; Guo et al., 2007; Nozaki et al., 2001; Namgung and Xia, 2001; Nath et al., 2001; Harper et al., 2001; Zou et al., 2002; Irving and Bamford, 2002; Barone et al., 2001). The roles of some of these kinases in neurodegeneration have been confirmed through the use of

specific kinase/pathway inhibitors (Raoul et al., 2006; Horiuchi et al., 2003). These kinases can be induced by various stimuli that lead to neurodegeneration. For example, release of ROS during secondary insults not only damage cellular lipids, proteins and nucleic acids, but also initiate redox-dependent MAP kinase signaling pathways that play key roles in mediating distinct cellular responses, including glial cell activation and neural cell apoptosis. Amongst such redox-dependent MAPKs are the p44/42, JNK, and p38, the latter two being particularly studied for their pro-apoptotic characteristics (Emerling et al., 2005; Karin and Gallagher, 2005; Shen et al., 2006; Sumbayev et al., 2005). It should be noted that although the ERK 1/2 is a pathway commonly associated with cell survival, in some systems including the CNS, it can also signal stress-mediated cell injury (Chiu et al., 2010; Zhuang et al 2007; Kyriakis and Avruch, 2001).

MAPKs are critically involved in the activation of microglia which plays an important role as immune cells in CNS (Watts et al., 2010; Matsushita et al., 2008; Yamasaki et al., 2007; Schieven, G. L, 2005; Zarubin and Han, 2005). Activation of MAP kinases have been reported to promote apoptosis and inflammatory responses in a variety of neurodegenerative diseases such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (Kim, 2010; Ferrer et al., 2005; Ekshyyan and Aw, 2004; Barone et al., 2001; Park et al., 2002; Zhu et al., 2002). Studies with the mouse model of ALS expressing mutant SOD1 suggest that MAPKs may play a role in the development and progression of this condition (Bendotti et al 2005). The kinases are activated in both glia and motor neurons in this disease hence suggesting an involvement in glial activation/inflammation and neurotoxicity (Xu et al., 2009; Holasek et al., 2005; Bendotti et al 2005; Tortarolo et al., 2003). In neurons, p38 MAPKs are widely implicated in apoptosis of neurons (Guo and Bhat, 2007; Wada and Penninger, 2004). MAPK activation has been described in several disease models that induce apoptosis such as sciatic nerve injury (Murashov et al.,

2001), cobalt chloride-induced (Zou et al., 2002), nitric oxide-induced (Atzori et al., 2001, Cheng et al., 2001 and Ghatan et al., 2000), superoxide-induced (Oh-hashii et al., 1999), and NMDA receptor agonist-induced apoptosis models (Tikka and Koistinaho, 2001). These findings suggest that MAPK kinases may represent a target of neuroprotective action not only in SCI but other disease models of neurodegeneration wherein inflammation and apoptosis are part of the disease pathogenesis (such as ALS, MS, Parkinson's and Alzheimer's disease).

Since MAPK pathways play both pro-apoptotic and pro-inflammatory roles in both neurons and glia cells, an important implication of the findings of our present study is that targeting of MAPK kinases may have dual benefit. Therefore quercetin's ability to inhibit MAPK activation may contribute to its ability to attenuate both neuroinflammatory responses and apoptosis in SCI. Moreover, the anti-apoptotic effect of quercetin coupled with its known anti-inflammatory role may be exploited to extrapolate the use of this drug in other models of neurodegeneration such as MS and ALS.

8.5 Other possible mechanisms of quercetin's actions and future directions of studies

In the previous paragraphs we demonstrated in more detail, possible mechanisms responsible for the therapeutic effects of quercetin on SCI. A considerable amount of work looked at the effects of quercetin on signaling mechanisms involved in apoptosis and delayed inflammatory reactions (including reactive gliosis) in traumatic SCI.

Quercetin showed neuroprotective effects by regulating the expression of apoptosis-related proteins such as caspase and cleaved PARP that resulted in the suppression of cell death during the pathologic process after SCI. On the other hand, it is also possible that quercetin

suppress neuronal and glial cytochrome C release from the mitochondria. This could be another aspect to explore in future studies.

There is some controversy on the identity of cell types undergoing apoptosis after SCI. This appears to depend on the severity of injuries, time points of analysis and specific area of spinal cord examined. Although in this study we did not examine each of the individual cell types undergoing apoptosis, it has been reported that macroglia (such as oligodendrocytes) form an important component of cell types that undergo delayed apoptotic process after SCI (Casha et al., 2001; Beattie et al., 2002). It is thought that apoptotic cell death of oligodendrocytes (which usually occurs weeks after SCI) is responsible for the induction of myelin degeneration and hence causing additional disturbances of axonal function of neurons that survived the injury. This delayed appearance of apoptosis in oligodendrocytes is believed to provide a therapeutic window for intervention that is way beyond the acute phase of injury (Li et al., 1999a). Furthermore, it has been shown that the application of p38 MAPK inhibitor prevents delayed progressive degeneration of oligodendrocytes following traumatic SCI (Horiuchi et al., 2003). Therefore, by inhibiting MAPK p38 in chronic SCI, quercetin may be inhibiting oligodendrocyte apoptosis. By inhibiting oligodendrocyte apoptosis the drug would consequently inhibit demyelination. The presence of oligodendrocyte could then promote myelination of denuded axons. In this case, it can be postulated that quercetin may be promoting axon survival by inhibiting oligodendrocyte apoptosis. In this study we examined dual immunocytochemical staining for p38 activation with GFAP (astrocyte marker) following SCI and found strong colocalization of p38 within the GFAP positive cells (Appendix 1). Since MAP kinases may play both pro-inflammatory and pro-apoptotic roles it can be deduced from this finding that p38 may be influencing both the process of reactive gliosis and apoptosis of glia cells (in this case

astrocyte). We did not examine dual staining involving oligodendrocyte but we think that this same process could be happening in oligodendrocytes and therefore this is an important aspect to explore in further studies. Another future direction would be dual staining with TUNEL (or other apoptosis markers) and various cell type markers.

It is known that oxidative stress resulting from secondary injury can trigger JNK and NFκB pathways by inactivating protein phosphatases (Morita K et al., 2001; Xu D et al., 2002). Phosphatases inactivate kinases by enzymatic removal of the phosphate groups (dephosphorylation). Therefore inactivation of phosphatases will prevent dephosphorylation of MAP kinases thereby indirectly promoting kinase activation. Although we did not investigate dual staining for activated MAPK JNK and nuclearly localized NFκB p65 it is possible that one of the mechanisms of quercetin's action maybe through this indirect promotion of protein phosphatase activity (hence MAPK inactivation). By inhibiting c-jun and NFκB activation, quercetin may be inhibiting protein phosphatase inactivation that would otherwise allow retention of phosphate groups on proteins. This is another possible mechanism for future exploration.

We do not completely exclude other possible roles of quercetin than the regulation of apoptosis and inflammation in the functional recovery from SCI. The systemic actions of quercetin may results in other possible effects. Quercetin might exert systemic neuroprotective effects by stimulating cells to release and increase synthesis of various trophic factors, such as basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and neurotrophin-3 (NT-3) (Spencer J.P, 2008, 2009; In Koo Hwang et al., 2009; Wu et al., 2000). Such trophic factors can contribute to tissue preservation after trauma (McDonald and Belegu, 2006). The ability of quercetin to stimulate production and release of trophic factors may have relevance not only in

the early wave of neural apoptosis, but also may contribute to the reduced number of apoptotic cells observed in the delayed wave of apoptosis after spinal cord injury. The expression of these factors can be explored in future studies. We believe that the intraperitoneal injection of quercetin used in this study could exert these systemic effects because (1) quercetin can pass through the blood-spinal cord barrier, (2) administration by this route could bypass the portal circulation hence reducing extensive metabolism of the drug, (3) the blood-spinal cord barrier is commonly broken after SCI.

The question remains whether quercetin treatment at 2 weeks after injury promote activation of pathways associated with trophic support or decrease activation of pathways associated with apoptosis. While this study looked at signaling mechanisms involved in neurotoxicity and apoptosis, it is very possible that quercetin could be influencing pathways that are associated with trophic support. Further studies need to be done in this direction.

My work has shown that there is also better white matter preservation in the treated animals. We observed that in animals treated at 2 weeks after injury, the ability to attain a BBB score of 8 or higher are associated with significant retention of white matter at the site of injury. Therefore, quercetin must either decrease pathways that cause cell damage or promote pathways that are trophic (i.e. could be preventing glia (e.g. oligodendrocyte) apoptosis, or promoting glia growth from precursor cells). Our results suggest the inhibition of apoptosis but the later possibility is also highly likely and therefore still needs to be addressed by further studies.

8.6 Summary and Conclusions

The recent progress of neuroscience has enabled various pathophysiological mechanisms of several neurodegenerative processes to be clarified and many potential candidates for

therapeutic targets have emerged. However, in the case of traumatic SCI when possible therapeutic interventions are designed, much of the focus is geared toward intervention at the acute stage of the disease. Hence, despite the fact that most patients with SCI are living with the condition in a chronic state, not many studies have looked at late intervention following traumatic SCI. Therefore, novel therapeutic methods based on modern basic neuroscience should be developed for chronic SCI.

The major aim of this study was to investigate the signaling mechanisms behind quercetin's actions in chronic SCI particularly its effect on various signaling pathways known to promote secondary insults. In the course of this study, we were able to investigate some of the mechanisms that underlie the biological actions of quercetin in spinal cord injury, with particular focus on the effect of quercetin administration on axonal transport, tissue sparing, reactive gliosis, and apoptosis, and on the effects of quercetin on the activated MAP kinases associated with neurotoxicity. The hypotheses and each of the objectives addressed in this study were upheld throughout the course of these experiments.

The first hypothesis stated that quercetin promotes locomotor functional recovery in chronic SCI which is associated with decreased cell damage and neural tissue sparing. This hypothesis was supported by both behaviour studies and histology studies (H and E, LFB and TB) and white matter quantification in spinal cord tissue sections. By means of behaviour analyses (BBB and incline plane scores), we were able to show that quercetin administration improved locomotor recovery. Histology studies showed better preservation of tissue structure with less cavity formation and better myelination and preservation of white matter content following quercetin treatment after SCI.

The second hypothesis states that the protective effect of quercetin in spinal cord injury is associated with decreased cell apoptosis. We investigated the molecular basis for the anti-apoptotic activity of quercetin in rat chronic SCI model by examining DNA fragmentation in apoptosis with the use of TUNEL method as well as the expression of apoptosis-related proteins (activated caspase 3 and cleaved PARP) by means of immunocytochemistry and Western blotting. We demonstrated that quercetin inhibited caspase 3 activation as well as PARP cleavage and DNA fragmentation, in rat SCI model, thereby providing the molecular evidence for its neuroprotective activity.

The third hypothesis states that quercetin improves axon function and decreases excess inflammatory responses in SCI and was also sustained by immunocytochemistry and Western blot analysis. Axon function, analyzed by examining accumulation of β -APP, showed better outcome in quercetin treated compared to the saline control. Moreover, by analyzing the expression of cellular markers ED1, OX-42 and GFAP, we were able to show that administration of quercetin significantly attenuated inflammatory processes including reactive gliosis in treated animals compared to saline control animals after SCI.

Lastly, the fourth hypothesis states that quercetin down regulates the phosphorylation of various MAPK kinases that are known to be implicated in inflammation, oxidative stress and apoptosis. This hypothesis was also validated by immunocytochemical and Western blot analysis. We were able to show that quercetin administration was associated with down regulation of MAP kinases that have been reported to promote tissue damage and apoptosis in the CNS. Our findings raise the possibility that delayed treatment could be rescuing neurons that had survived over the first two weeks following injury by inhibiting reactive gliosis and

apoptosis of glia cells. If this is the case, then quercetin may be acting via selective inhibition of kinase pathways that have been shown to be involved in neural cell injury and apoptosis.

In summary, my results indicate that the therapeutic actions of quercetin probably occurs by multiple mechanisms including reducing inflammation, reactive gliosis and inhibiting apoptotic cell death of neural tissue (including neurons, astrocytes oligodendrocytes, and microglia) all leading to an improvement of locomotor functional recovery. These data raise the exciting possibility that quercetin may also be able to reduce secondary pathological events and thus improve functional outcome after traumatic spinal cord injury in humans.

8.7 Significance

This study is important not only because it demonstrates the neuroprotective effect of quercetin administration in chronic spinal cord injury, but also because it indicates some of the potential mechanisms whereby quercetin may exert its neuroprotective effects in vivo. These findings provide a basis for further exploration of the mechanisms underlying the neuroprotective effects of quercetin. Furthermore, and of potential clinical importance, is that quercetin was effective when it was administered 2 weeks after the injury raising the possibility of some positive outcome even when treatment initiation is delayed.

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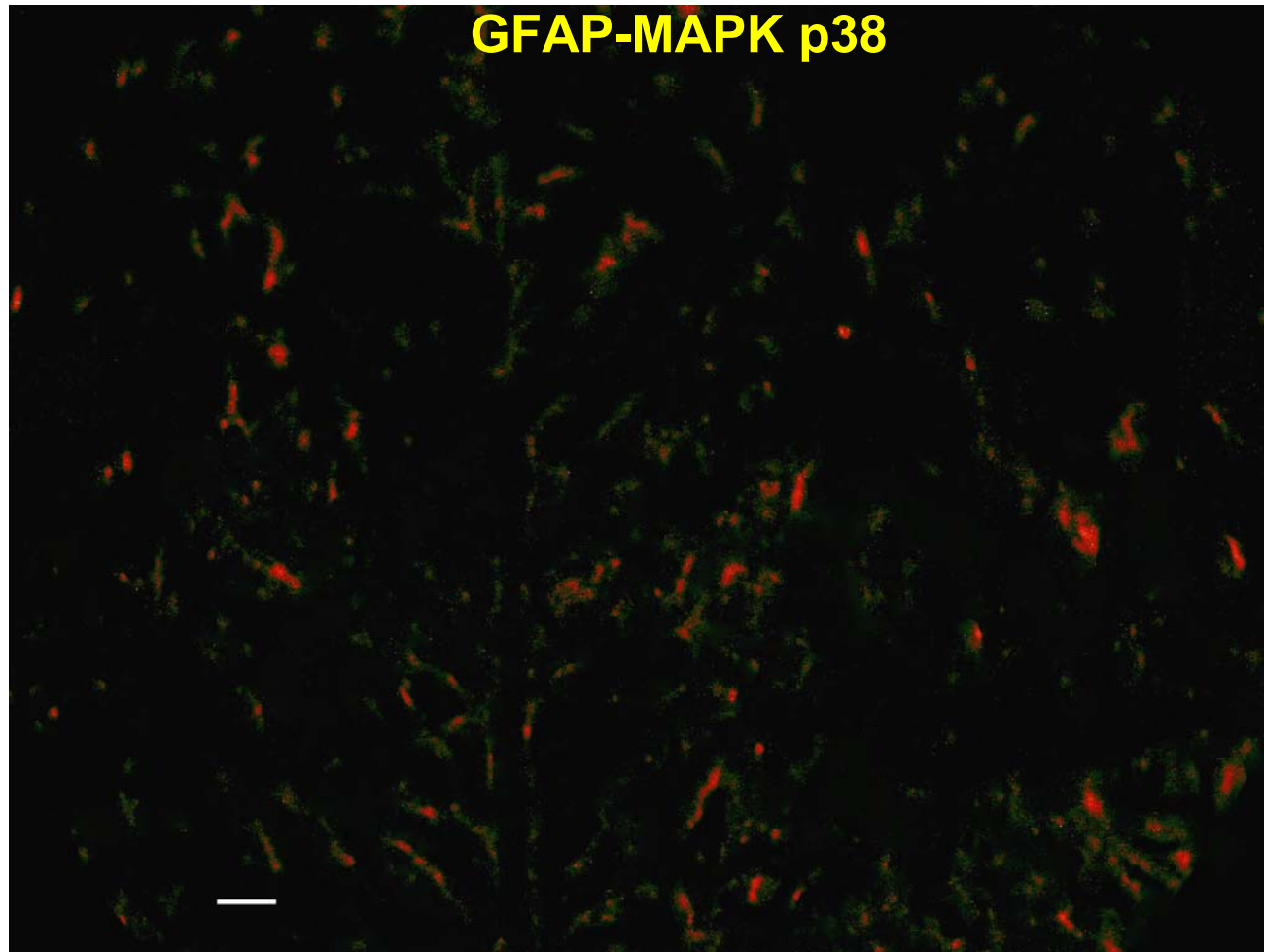
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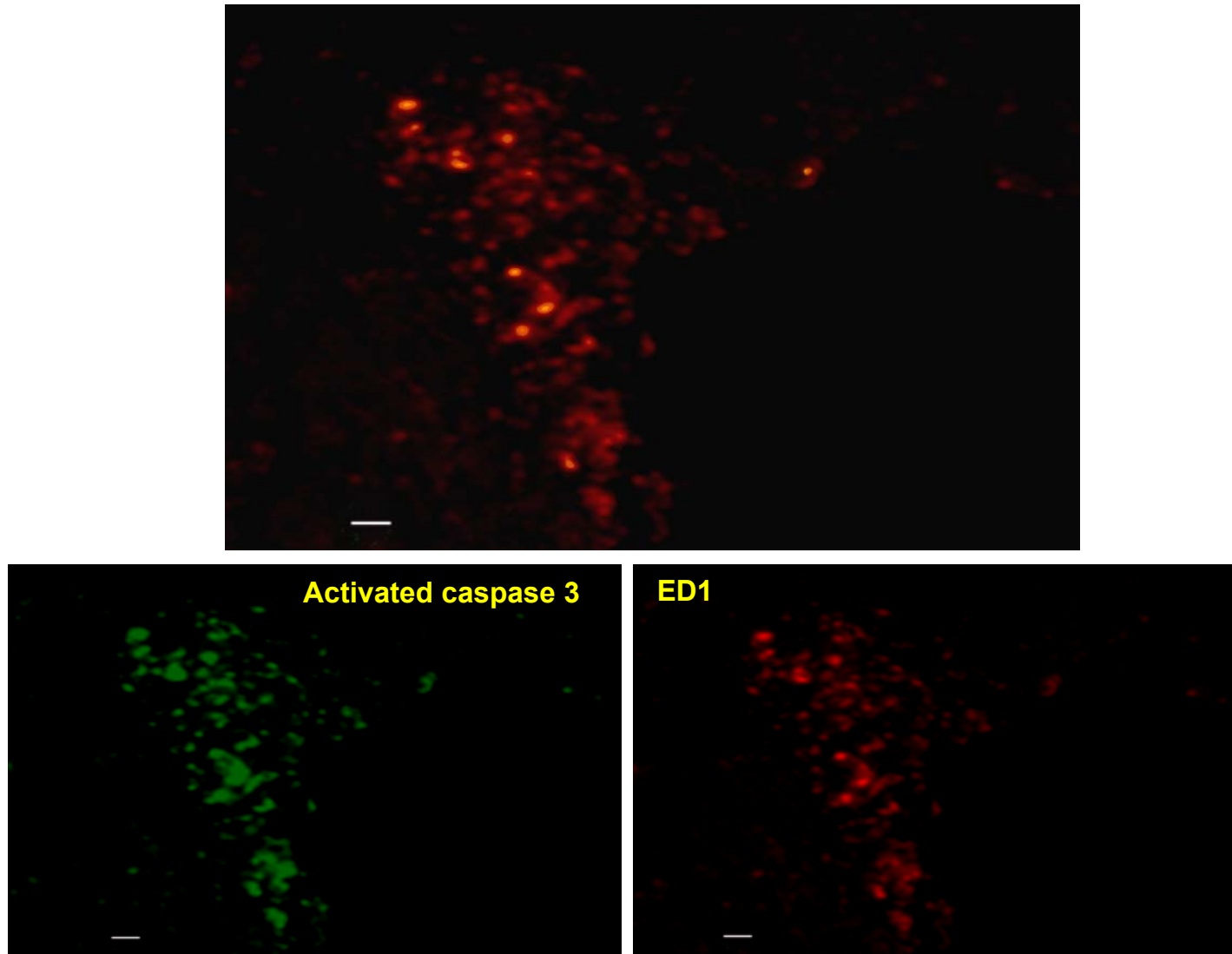
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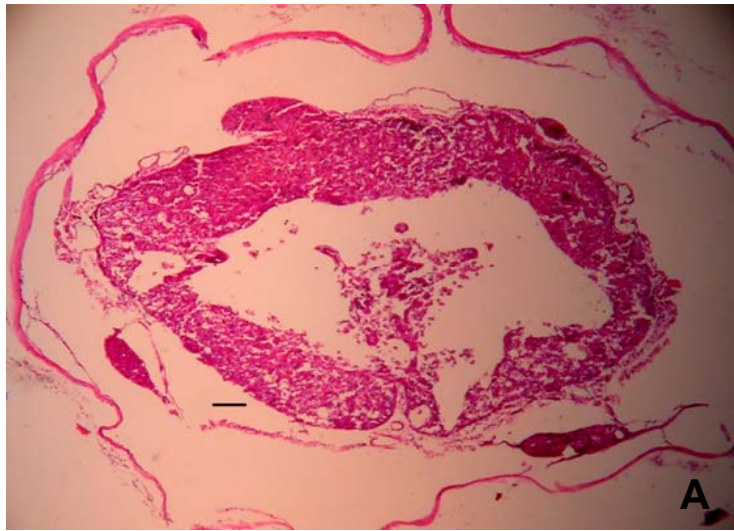
APPENDICES



APPENDIX 1: Photomicrograph of double immuno-staining for phospho-MAPK p38 (green) and GFAP (red) using spinal cord sections harvested at eight weeks post surgery in a saline control animal. Most GFAP positive cells are co-localized with phospho-MAPK p38 indicating increased MAPK p38 activity during gliosis (activated astrocytes). Scale bar = 50 μ m



APPENDIX 2: Photomicrograph of double immuno-staining for activated caspase 3 (green) and ED1 (red) using spinal cord sections harvested at eight weeks post surgery in a saline control animal. There is a strong activated caspase3 and ED1 positive cell co-localization indicating increase caspase activity during gliosis (activated microglia). Scale bar = 50 μ m



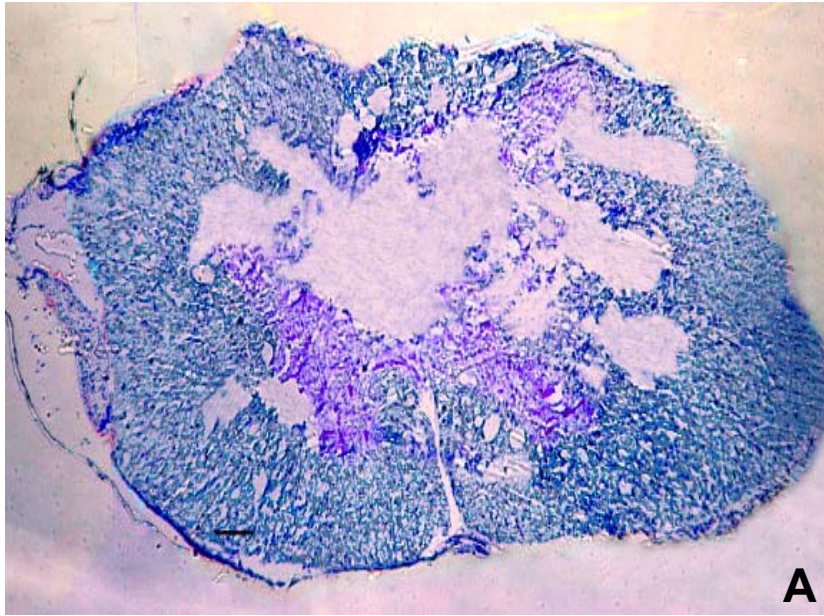
Saline



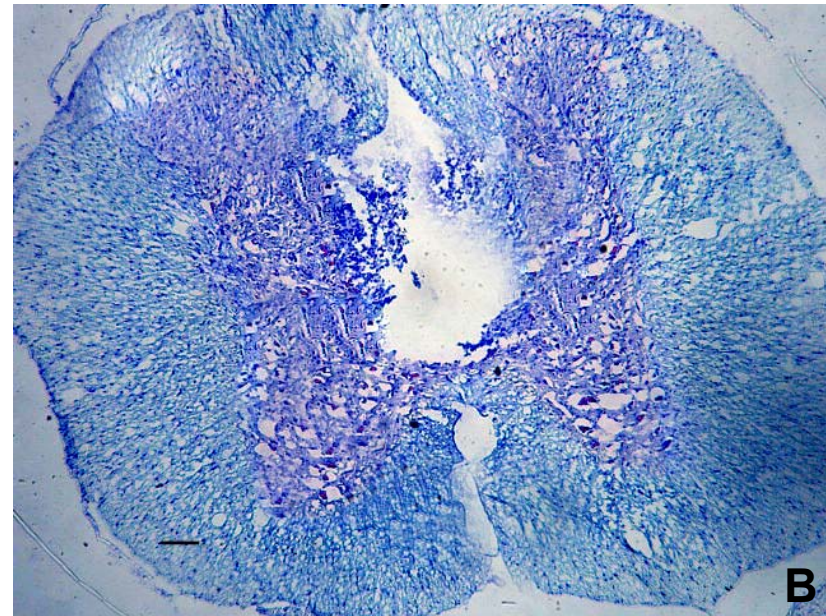
Quercetin



App. 3 (a)



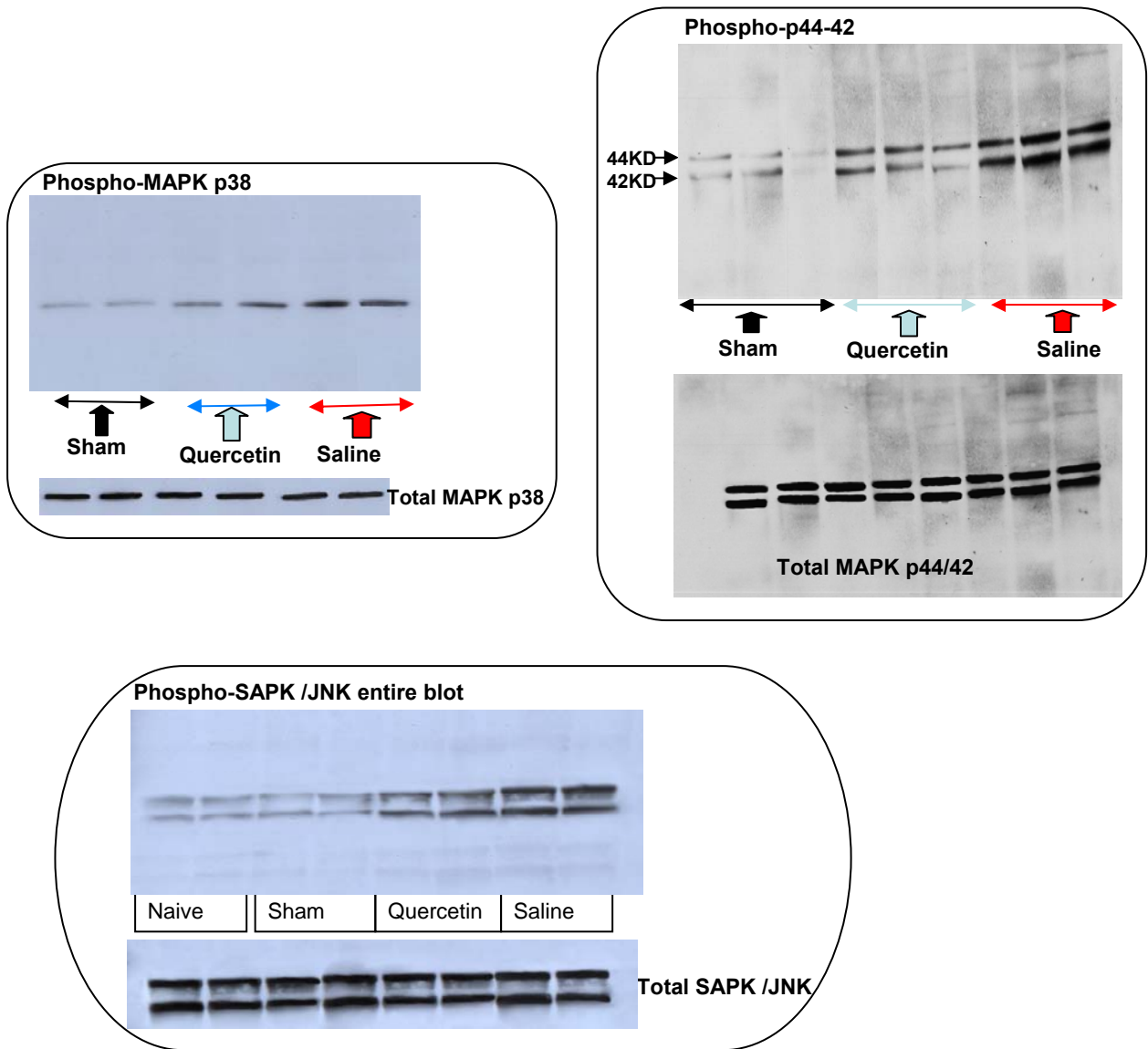
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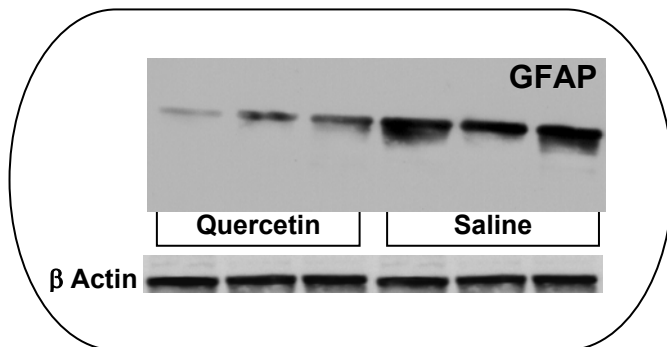
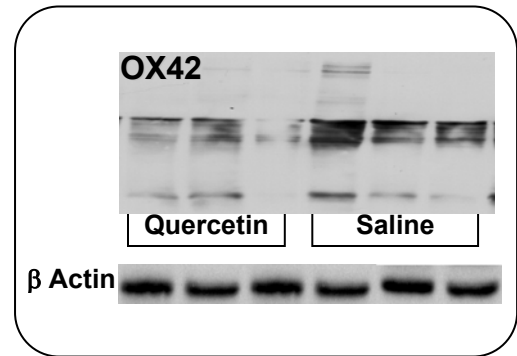
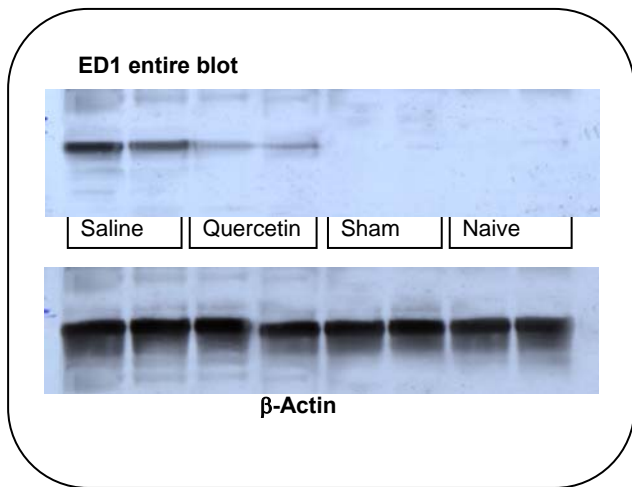
Quercetin

App. 3 (b)

APPENDIX 3 (a) (b): Photomicrographs of H and E (a) in saline control A and quercetin treated B, sham operated, using sections from spinal cord tissue harvested at eight weeks post surgery (scale bar = 100 μ m). LFB (b) staining in saline control A, and quercetin treated B, using sections from spinal cord tissue harvested at eight weeks post surgery. Scale bar = 80 μ m.



APPENDIX 4A: Western blots for phospho-MAPK p38, p44/42 and the entire original blot of phospho-SAPK (shown in fig. 7. 12a) which shows activated SAPK/JNK expression in naïve animals. Experiments were done using spinal cord tissues harvested at eight weeks post surgery.



APPENDIX 4B: Western blots showing entire original blot of phospho ED1 (shown in fig. 7. 6a) and other representations of OX-42 and GFAP. Analyses were done from spinal cord tissues harvested at 8 weeks post surgery.

APPENDIX 5

Hematoxylin and Eosin Staining Protocol (Harris Hematoxylin)

Preparation of Solutions:

Harris Hematoxylin

100 g	Aluminum Potassium Sulfate ($\text{Al K}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$)_OR_Aluminum Ammonium Sulfate ($\text{Al NH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$)
1000 ml	Distilled Water
5 g	Hematoxylin (C.I. No. 75290)
50 ml	Absolute Alcohol (i.e. 100% Ethanol)
0.5 g	Sodium Iodate
30 ml	Glacial Acetic Acid

Dissolve the aluminum sulfate in distilled water and then dissolve hematoxylin in absolute alcohol and add to the aluminum potassium sulfate solution.

Add sodium iodate and leave solution to sit overnight. Next day, add acetic acid and it's ready to use.

Eosin

Stock Solution: 1.25% Eosin Y (C.I. No. 45380) in 70% Ethanol.

Add 1 ml of Glacial Acetic Acid to every 100 ml.

Working Solution: 1 part Stock Solution to 4 parts 70% Ethanol

H and E Staining:

NB: Steps 1 to 6 involves deparaffinising and hydrating paraffin embedded sections. For frozen sections, leave to equilibrate at room temperature for 1 hr then begin staining at step 6.

1. 3 minutes each: 2 changes of Xylene
2. 1 minute: Xylene / Absolute Alcohol
3. 1 minute each: 2 changes of Absolute Alcohol
4. 1 minute: 95 % Ethanol
5. 1 minute: 70% Ethanol
6. 1 minute: Tap Water
7. Rinse: Distilled Water
8. 3 - 5 minutes: Harris Hematoxylin
9. Wash: Tap Water (several changes)
10. 2 dips: Acid Alcohol (0.5% HCl in 95% Ethanol)
11. Wash: Tap Water (several changes)
12. 5 seconds: Saturated Aqueous Lithium Carbonate
13. 3 minutes: Running Tap Water
14. Rinse: Distilled Water
15. 1 minute: Eosin
16. 1 minute: 70 % Ethanol
17. 1 minute: 95 % Ethanol
18. 1 minute: Absolute Alcohol 1
19. 1 minute: Absolute Alcohol 2
20. 1 minute: Absolute Alcohol / Xylene
21. 1 minute each: 3 changes of Xylene
22. Leave slides in xylene until ready to coverslip then coverslip slides in a permanent mounting medium.

APPENDIX 6

Toluidine Blue (pH 3.0) Staining Protocol

Preparation of Solution (1% Toluidine Blue in 1% Sodium Borate):

1 g. Sodium Borate Decahydrate (Borax) → $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$

1 g. Toluidine Blue (C.I. 52040)

100 ml Distilled Water

Adjust the toluidine blue stain to a pH of 3.0 in order to amplify metachromatic staining.

Staining:

1. Paraffin sections were deparaffinised as outlined in steps 1 to 6 of appendix 5.
2. Frozen sections from the freezer were left to equilibrate at room temperature for 1 hr.
3. The sections were then fixed in room temperature methanol for thirty minutes.
4. The slides were stained for about 1 minute, after which the stain was washed off with tap water.
5. The slides were placed then left to completely dry, then coverslipped with a permanent mounting medium.

APPENDIX 7

Luxol Fast Blue plus Nissl Counterstain (with Cresyl Violet Acetate) Protocol (Taken from “Laboratory Notes in Histological Technique”-Anatomy 412B 1982)

Preparation of Solution (Electron Microscopy Sciences Inc Hatfield, PA; #: 26681):

Luxol Fast Blue Solution:

0.1 g Solvent Blue 38-practical grade (*Sigma S-3382*)

100 ml 90% ethanol

0.5 ml concentrated glacial acetic acid

Filter before use. Keep used stain in separate container and replace after one week or sooner if stain is frequently used.

Lithium Carbonate Solution:

0.5 g Lithium carbonate

100 ml distilled water

Cresyl Violet Solution:

0.2 g Cresyl Violet Acetate (*Sigma C-1791*)

150 ml distilled water

Filter before use.

Buffer Solution (0.1M, pH 3.5):

94 ml 0.1 M acetic acid (6 ml glacial acetic acid /1000 ml distilled water)

6 ml 0.1 M Sodium acetate ($C_2H_3O_2 Na$) (13.6 g sodium acetate/1000 ml distilled water)

Working Solution:

150 ml Buffer solution

18 ml Cresyl violet solution

Use fresh lithium carbonate and working cresyl violet solutions daily. Replace any that appear cloudy or yellow.

Staining:

1. Deparaffinize and hydrate to 70% alcohol (see steps 1 to 6 of appendix 5).
2. Leave in slides in Luxol Fast Blue Solution and place the staining dish in 37°C warm bath or oven overnight.
3. Rinse off excess stain with distilled water.
4. Place slides in fresh distilled water.
5. Begin differentiation by immersion of the slides singly in Lithium Carbonate Solution for 30 seconds.
6. Continue differentiation in 70% alcohol until the gray matter is clear and white matter is sharply defined.
7. Rinse in distilled water for 1 minute
8. Check microscopically for desired stain intensity. Repeat the differentiation if necessary starting at step 5.
9. When differentiation is complete, place in distilled water.
10. When all slides have been collected in distilled water, add fresh distilled water.
11. Counterstain in Cresyl Violet Acetate working solution for 12 minutes.
12. Rinse for 1 minute in 70% alcohol in 95% alcohol.

13. Continue the dehydration through two changes each of absolute ethyl alcohol first time 3 minutes then 2 minutes.
14. Wash 1 minute in Xylene/absolute then 2 changes in xylene, for 2 minutes.
15. Mount and coverslip with a permanent mounting medium.