Therapeutic potential of curcumin in a spinal cord injury model: Local application versus dietary supplement

Sarah Kamel Farahat Abu hussein

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THERAPEUTIC POTENTIAL OF CURCUMIN IN A SPINAL CORD INJURY MODEL: LOCAL APPLICATION VERSUS DIETARY SUPPLEMENT

A Thesis Submitted to

The Biotechnology Graduate program

In partial fulfillment of the requirements for
the degree of Master of Science

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Under the supervision of
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July 2017
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DEDICATION

To the most important people in my life

Dad, I am blessed for being your daughter, I miss your presence.
Mum, you are all the love in my life; none of this would have been possible without your faith in me
My Husband, Thank you for always being there for me and for truly believing in me
My Brother and Sister, Thank you for being extremely supportive and caring all the way
My Sons, you are the air that I breathe, looking into your eyes makes everything look perfect
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ABSTRACT

Spinal cord injury is a debilitating disability that is characterized by a sequence of tragic events that occur following the primary impact aggravating the condition, collectively called secondary spinal cord injury. Oxidative damage and inflammatory surge are two hallmarks of the secondary spinal cord injury cascade. Curcumin is a polyphenolic compound extracted from the rhizome of *Curcuma longa* that has been well known to possess antioxidant and anti-inflammatory properties. The objective of this study was to evaluate the potential of curcumin as an antioxidant and anti-inflammatory agent following spinal cord injury, and to compare its therapeutic effects following local application directly to the injury versus its effect when given as a dietary supplement in a spinal cord hemisection model at T9-T10 level of the spinal cord. Female Sprague Dawley rats were randomized into a control group, injury groups (1 day and 7 days), local treatment groups single dose of *Curcuma longa* extract immediately on the injury site (1 day and 7 days) and a Dietary supplement group. Crude Curcumin was added to the animals’ feed (10% of daily feed) one week before and week after injury. Oxidative stress parameters used for detection the effect of Curcumin before and after treatment were Malondialdehyde (MDA) by Thiobarbituric acid assay (TBA) and total antioxidant capacity (TAC). Expression of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) was detected using Enzyme Linked Immunosorbent Assay (ELISA). Our results show that at 7 days, although Dietary supplement was effective in increasing TAC levels and lowering TNF-α expression levels, yet it did not affect MDA levels (IL-6 data not measured). Local treatment regimen has shown to be more effective on all four parameters measured as the 7 days. Our results demonstrate that local Curcumin application directly on the injury site might be more efficacious in alleviating oxidative damage and reducing inflammation following spinal cord injury. Further analysis is needed to evaluate the effect of Dietary treatment regimen on IL-6 and detect the effect of different Curcumin treatment regimens on other antioxidant and anti-inflammatory markers to investigate the role of curcumin in alleviating oxidation and inflammation.
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<tr>
<td>AACR</td>
<td>American Association for cancer research</td>
</tr>
<tr>
<td>AMPARs</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor</td>
</tr>
<tr>
<td>AN</td>
<td>Athymic nude</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>ASIA</td>
<td>American Spinal Injury Association</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BSCB</td>
<td>Blood-spinal cord barrier</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CO₃⁻</td>
<td>Carbonate Free Radical</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin Sulphate Proteoglycan</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic Acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DSPG</td>
<td>Dermatan Sulphate Proteoglycan</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERKs</td>
<td>Extracellular signal regulated Kinases</td>
</tr>
<tr>
<td>GABAₐ</td>
<td>Gamma-Amino Butyric Acid</td>
</tr>
<tr>
<td>GAP 43</td>
<td>Growth Associated Protein 43</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GCL</td>
<td>Glutamate Cysteine Ligase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Stimulating Factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GLU</td>
<td>Glutamate</td>
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<tr>
<td>GM-1</td>
<td>mono-sialo-tetra-hexosyl-ganglioside</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>hESC</td>
<td>Human Embryonic Stem Cells</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-Hydroxynonenal</td>
</tr>
<tr>
<td>HO-1</td>
<td>Hemeoxygenase 1</td>
</tr>
<tr>
<td>HO</td>
<td>Heterotopic ossification</td>
</tr>
<tr>
<td>HSC</td>
<td>Hepatic stellate cells</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan Sulphate Proteoglycan</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia Reperfusion</td>
</tr>
<tr>
<td>IFN Ɣ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1β</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>IL-13</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>JAK-STAT</td>
<td>Janus Kinase/Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Keap 1</td>
<td>kelch-like ECH associated protein 1</td>
</tr>
<tr>
<td>KSPGs</td>
<td>Keratan Sulphate Proteoglycans</td>
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<tr>
<td>LOX</td>
<td>Lipoxygenase</td>
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<td>LPS</td>
<td>Lipopolysaccharides</td>
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<tr>
<td>MAG</td>
<td>Myelin associated glycoprotein</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
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MMP-9  Matrix Metallopeptidase 9
MP     Methylprednisolone
NF-κB  Nuclear Factor kappa B
NG2    Neural/Glial antigen 2
NgR1   Nogo Receptor 1
NOS    Nitric oxide synthase
NQO1   Nicotinamide Adenine Dinucleotide Phosphate Quinine Oxidoreductase
Nrf2/ARE Nuclear Factor E2 Related Factor-2/ Antioxidant Response Element
OPC    Oligodendrocyte progenitor cells
PAMP   Pathogen associated molecular pattern
PBS    Phosphate Buffered Saline
PGE2   Prostaglandin E2
PMA    Phorbol 12-Myristate 13-Acetate
PON    Peroxynitrite
PPAR-γ Peroxisome Proliferator- Activated Receptor Gamma
ROS    Reactive oxygen species
SCI    Spinal cord injury
SD     Sprague Dawley
SLCP   Solid Lipid Curcumin Particle
SOD    Superoxide dismutase
STAT   Signal transducer and activator of transcription
TAC    Total Antioxidant Capacity
TBA    Thiobarbituric Acid Assay
TBI    Traumatic brain injury
TGF β  Transforming Growth Factor Beta
TLR4   Toll like receptor 4
TNF    Tumor necrosis factor
TNFR1  Tumor Necrosis Factor Receptor 1
TRPA-1 Transient Receptor Potential Ankyrin -1
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CHAPTER 1: LITERATURE REVIEW

1.1. Spinal Cord Injury

1.1.1 Introduction
Spinal cord injury (SCI) is a debilitating tragic disability that affects a lot of people worldwide. About 2.5 million people have SCI with about 130,000 new injuries affecting people annually (Thuret et al. 2006). SCI has been described in an ancient Egyptian physician textbook called “The Edwin Smith papyrus” since 1700 BC as an “ailment not to be treated” (Porter, 2001)

It influences not only the life of the individual but also the surrounding family members socially, emotionally and financially. The cost of being enrolled in a clinical trial per person is about 50,000 to 100,000 dollars in Europe and United States. On the other hand the cost of preliminary phase 2 human efficacy trials for a drug is about 5 to 10 million dollars (Tator, 2006). In Spite of all of this, available treatments give modest benefit that’s why more effort is needed to come up with more effective SCI treatment regimens (Ali and Bahbahani 2010)

Many of the complications of SCI are permanent and eventually cause serious consequences, including; respiratory complications which are a frequent cause of death after SCI (Berney et al., 2011), Bladder dysfunction (Hagen, 2015), and heterotopic ossification (HO) (Banovac et al., 2004), which leads to reduction in joint motion range.

Unfortunately, to date the approaches taken to handle or treat SCI can be still regarded as sedative, or palliative approaches trying to prevent further progression, handle dysautonomia and complications of loss of sensory abilities and aiming at teaching patients on how to cope up with their disabilities (Jablonska et al. 2010, Silva et al., 2014).
1.1.2 Incidence and Prevalence:

In most countries, SCI patients are below 30 years of age (Singh et al., 2014; Rhee et al., 2013). A higher male to female ratio is demonstrated and traffic accidents were the most known cause for SCI followed by falls in elderly. The highest SCI prevalence was (906/million) in the United States of America and lowest (250-280/million) in France and Finland (Singh and Tetreault et al., 2014).

1.1.3 Anatomy of the Spinal Cord:

The spinal cord extends from the medulla oblongata in the brainstem through the foramen magnum of the skull till lumbar vertebra L1 in humans or L3 in rats. The spinal cord provides connection between the brain and the peripheral nerves. It is protected by the bony vertebral column and by the three meninges namely: Dura, Arachnoid and Pia matter. Additional protection is provided by the subarachnoid space (between pia and arachnoid) which is filled with cerebrospinal fluid as well as, the epidural space between dura and periosteum; which is filled with adipose connective tissue and loose fibrous tissue (Vander et al., 2001).

The gray matter is located centrally and is composed of neurons, interneurons, dendrites and cell bodies of motor neurons, glial cells and the entering fibers of the sensory neurons. The white matter on the other hand is mainly composed of myelinated axons which are organized in functional groups called tracts; descending to the periphery or ascending to the brain transmitting information in both sides, as well as propriospinal (local) tracts which connect and coordinate different levels of the spinal cord. These tracts are regarded as a crucial communication means between the brain and the spinal cord (Silva et al., 2014).
Descending tracts (pyramidal or extrapyramidal) control motor functions as shown in figure 1. Pyramidal tracts are so named due to the fact that they decussate (cross from one side to the other) in the medulla. These tracts terminate at the lower motor neurons in the grey matter of the spinal cord. Extrapyramidal tracts include: rubrospinal, reticulospinal, tectospinal and vestibulospinal tracts. These tracts are mostly responsible for complex movements, fine locomotion tuning, reflexes and postural control.

Ascending tracts shown in figure 1 convey information from the periphery to the cerebral cortex. The dorsal column fibers carry proprioceptive, vibratory senses and fine touch. The anterolateral system (anterior & lateral spinothalamic tracts) carries crude touch, temperature and pain. The spinocerebellar tracts aid in fine tuning and coordination of motor tasks and carry unconscious proprioceptive data (Lee and Thumbikat, 2015).

Crossing of fibers carrying proprioception, motor function and light touch in the medulla means that the SCI patient will experience an ipsilateral (same side) deficit while decussation of fibers conveying information for temperature and pain occurs at their vertebral level of entrance into the spinal cord leading to a contralateral deficit upon injury (Lee and Thumbikat, 2015).
Figure 1: Ascending and Descending tracts of the Spinal Cord. Reproduced from Lee and Thumbikat 2015 with permission.

Axons of afferent (sensory) neurons enter the spinal cord via the dorsal root with their cell bodies present in the dorsal root ganglion, while those of efferent (motor) neurons leave the spinal cord through ventral roots. Eventually dorsal and ventral roots combine to form the mixed spinal nerve (motor, sensory and autonomic) which exit from 31 vertebral levels (Cervical-8, Thoracic-12, Lumbar-5, Sacral-5 and one coccygeal nerve) (Silva et al., 2014).

1.1.4 Pathophysiology of SCI

1.1.4.1 Primary Injury:

SCI pathophysiology is biphasic in nature entailing a primary and a secondary phase. The primary mechanical injury leads to a cascade of secondary damage involving vascular, cellular and biochemical events (Simon et al. 2009; White-Schenk et al., 2015). Traumatic SCI was classified by Bunge and colleagues as “a contusion with cavity formation, massive compression or laceration” (Bunge et al., 1997).
Vascular ischemia is not an uncommon cause for SCI especially as a surgical complication during the surgery for aortic aneurysm repair (Hollier et al., 1992) where spinal cord ischemia might result from prolonged aortic cross clamping or ligation of lumbar or intercostal arteries causing ischemia (Anwar et al., 2016), that might eventually lead to paraplegia (Shimizu and Yozu 2011).

This primary phase is marked by a series of events shown in figure 2 as spinal shock, systemic hypotension, ischemia, damaged plasma membrane and ionic imbalance (Oyinbo, 2011).

![Figure 2: Pathophysiology of SCI.](image)

A primary injury caused by impact or compression is followed by a secondary injury cascade that entails a series of cellular, molecular and biochemical events that contribute to worsening the primary injury. Reproduced from Esposito and Cuzzocrea 2011 with permission.

1.1.4.2 Secondary injury:

The secondary injury starts to take over following the primary phase and it can last for weeks (Wilson et al., 2013), the two major events that largely contribute to the secondary injury are inflammation and oxidative stress, which lead to necrosis, immune system response and apoptosis (Johnson et al., 2005), neuro-inflammation, free radicals formation, breakage of the blood-spinal cord barrier (BSCB), ischemic dysfunction, neuronal injury
and glial scar formation which limits the SCI recovery (Ormond et al., 2014). It also includes other functional problems, such as bladder, rectal and anal incontinence due to autonomic dysfunction, neuropathic pain and impotence (Singh and Tetreault et al., 2014).

Unfortunately in most cases the aggravating effects of the secondary injury commence before expert intervention starts (Oyinbo 2011). That’s why treatment strategies are focusing on combating this sequence of events (Fehlings and Nguyen 2010), and new strategies would help enhance axonal regeneration and neuronal function, and restore the disrupted functions that follow the injury.

Finally the chronic phase commences which may last for years post SCI, causing continued demyelination, apoptosis and cavitation well as syringomyelia which lead to permanent neurological impairment (Yiu and He 2006; Oyinbo, 2011).

1.1.5 Main Events in Secondary Spinal Cord Injury

1.1.5.1 Inflammation

Following SCI, the net result of the immune system response through its cellular (immune cells) and molecular (regulatory proteins) components is inflammation (Oyinbo, 2011). Immune cells secrete pro-inflammatory cytokines as tumor necrosis factor α (TNF-α), interleukin 1β and Interleukin-6 increasing the extent of inflammation, to remove cellular debris, unfortunately, over-activation of this inflammatory response aggravates the injury and damages the healthy tissues (Oyinbo, 2011).

The role of some immune cells in SCI is regarded as controversial (Donnelly and Popovich, 2008). Macrophages and microglia are thought to be a key component for neural regeneration while others suggest that these cells are responsible for oligodendrocytes damage (Merrill et al., 1993), demyelination and neuronal death (Rhoney et al., 1996). The
infiltration and activation of glial cells and leukocytes becomes the main source of reactive oxygen species (ROS) (Bains and Hall, 2012). The inflammatory cascade following SCI is shown in figure 3.

Figure 3: Inflammatory environment following SCI. The primary injury causes hemorrhage and increase in vascular permeability. Leukocyte extravasation and infiltration then occurs breaking up inter-cellular proteins and extracellular matrix. ROS are then released from activated microglia and leukocytes causing protein modification, lipid peroxidation and gene expression modulation eventually leading to cellular and tissue damage causing necrosis and apoptosis in neuronal and glial cells within the spinal cord parenchyma. Yet, the anti-inflammatory subsets of glial cells and leukocytes potentiate anti-inflammatory mechanisms. Reproduced from Anwar et al., 2016 with permission.

1.1.5.2 Neutrophils and Macrophages:

Following SCI, neutrophils arrive first at the injury site guided by the vascular endothelial cells. They enhance recovery by removing tissue debris and microbial invaders, and recruit macrophages (Kigerl et al., 2006). They also release proteases, free radicals, cytokines, and
activate other inflammatory and glial cells ultimately causing neuronal and glial toxicity and death (Joy et al. 2005, Shamash et al., 2002).

Depletion of neutrophils (Noble et al., 2002), inhibition of their adhesion (Hamada et al., 1996) or blocking of their related enzyme proteolytic activities (Noble et al., 2002) were correlated with neuroprotection (Kigerl et al., 2006).

Neutrophils and macrophages synthesize a variety of pro-inflammatory cytokines e.g. TNF-α and IL-1β as part of their function in normal cellular communication (Probert and Selmaj, 1997) ; however, the sustained release of these molecules in SCI triggers inflammation and disrupts the cytokine release pattern, potentially exaggerating glutamate excitotoxicity causing neuronal and oligodendrocyte death (Shamash et al., 2002).

Glutamate is the main excitatory neurotransmitter within the CNS (central nervous system), and excess amounts of it are normally removed by astrocytes and to a lesser extent by microglia (Rimaniol et al., 2000). However, after SCI its metabolism by astrocytes is disturbed and its clearance is prohibited by tumor necrosis factor alpha (TNF-α) or interleukin 1beta (IL-1β) (Takahashi et al., 2003). Neurons and oligodendrocytes are particularly sensitive to glutamate excitotoxicity as they demonstrate high numbers of glutamate receptors (Oyinbo, 2011). Blocking TNF-α or IL-1β enhances neuroprotection in SCI, traumatic brain injury (TBI) and stroke models (Sharma et al., 2003). Moreover, interleukin-6 (IL-6) overexpression was shown to potentiate leukocyte infiltration and impair both locomotor activity and axonal regrowth (Lacroix et al., 2002).

Stellwagen and colleagues demonstrated that TNF-α causes an elevation in α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPARs) specifically non-glutamate (non-GLU2-AMPARs) through tumor necrosis factor receptor 1 (TNFRI) rendering them more calcium permeable. At the same time TNF-α causes fewer expression of surface gamma-aminobutyric acid (GABA_A) receptors, consequently causing alleviation
in the inhibitory synaptic strength and enhancing excitotoxicity (Stellwagen et al., 2005). Potassium (K+) and Calcium (Ca2+) may also have a role in neuronal destruction (Jung et al., 2013).

Neutrophils and Macrophages also release superoxide and Nitric oxide which both react and combine releasing the highly toxic species, peroxynitrite (Xiong et al., 2007). After injury, depolarization causes opening of voltage dependent Ca2+, K+ and sodium (Na+) channels followed by Ca2+ overload leading to peroxynitrite (PON) derived free radicals production as shown in figure 4. These peroxynitrite radicals react with amino acids causing protein Carbonylation and damage the unsaturated fatty acids causing cellular damage (Xiong et al., 2007).

### 1.1.5.3 Lymphocytes:

After injury, Activated Lymphocytes carry out an autoimmune response that exaggerates the effect of macrophages causing more toxicity to the neurons and damage to the blood-brain barrier (Sriram and Rodriguez, 1997). T-lymphocytes appear to play a dual role in injury and repair depending on the surrounding microenvironment and the signals that recruited them (Trivedi et al., 2006). Chemokines are responsible for T-cell migration and activity modulation at inflammation sites (Sallusto et al., 2000). Some studies correlated their activation with increased cytokine release (Jones et al., 2005), fibrosis and scarring (Wynn, 2004).
Figure 4: Oxidative damage following SCI. Calcium overload is caused via glutamate receptor and voltage dependent channels. Mitochondrial oxygen (O2) uptake causes nitric oxide synthase (NOS) activation and eventually peroxynitrite radicals are formed causing damage and promote cytoskeletal proteins proteolysis and neurodegeneration. Reproduced from Bains and Hall 2012 with permission.

Neuro-antigen reactive T and B lymphocytes that act against myelin protein as Myelin Basic Protein (MBP) have noticeable pathological potential in Multiple sclerosis (Von Bűdingen et al., 2001). Transgenic rats and mice vaccinated to enhance MBP-reactive T cells, were shown to cause axonal impairment and demyelination causing neuronal loss (Jones et al., 2004).
Athymic nude (AN) rats manifested better locomotor activity than injured Sprague Dawley rats (SD) one week post injury in a study aiming at detection of T lymphocyte role in SCI. Moreover, injured SD rats showed acute elevation in death related genes suggesting a secondary wave of damage caused by T-cells. This suggests that a combination of T-cell inhibition and other neuroprotective strategies might have a promising therapeutic potential (Satzer et al., 2015).

Nevertheless, Schwartz and colleagues propose a protective role for T-cells specifically reactive to MBP in Lewis rats, in enhanced neuroprotection after spinal cord contusion or crushing optic nerve injury, but not in complete transaction model (Schwartz and Kipnis 2001).

B-lymphocytes also present in the injury site within hours post SCI and last for up to 1 week (Popovich et al., 2001). Enhanced activation of B cells was also evident following SCI, with elevated CNS antibodies in the serum of SCI individuals (Hayes et al., 2002) and in a mouse model of spinal cord contusion injury (Ankeny et al., 2006). B-lymphocytes also enhanced demyelination in an experimental allergic encephalomyelitis model (Chavarria and Alcocer Varela 2004).

1.1.5.4 Microglial cells:

Microglial cells exist as two main phenotypes, M1 the pro-inflammatory phenotype and M2 the anti-inflammatory phenotype which are two polarized states whose activation depends on external signals (Durafourt et al., 2012). Following SCI, microglia sense the initiation of tissue damage via toll-like receptors (Heiman et al., 2014), and undergo morphological changes, they attain an amoeboid morphology rather than the ramified appearance they maintain in their Quiescent state (Lee et al., 2007; Witcher et al., 2015).
Following SCI, M1 phenotype is activated and initiates a series of damaging side effects to neurons, axons, oligodendrocytes (Nguyen et al., 2012). This cascade is mediated through various signaling pathways, such as, inflammatory cytokines (TNF-α, IL-6) (Pineau and Lacroix, 2007), ROS (Barger et al., 2007), Chemokines (Mantovani et al., 2004) and Glutamate (Barger et al., 2007). These cytokines are expressed within two hours following injury (Pineau and Lacroix, 2007).

Moreover, the M1 phenotype has been found to express cell surface and intracellular receptors such as, CD45 (lymphocyte common antigen), CD11b (integrin family member) and inducible nitric oxide synthase 2 (iNOS2) which aids in the pro-inflammatory mechanism. On the other hand M2 phenotype releases anti-inflammatory cytokines as interleukin 4 (IL-4), interleukin 10 (IL-10) and interleukin 13 (IL-13) (Witcher et al., 2015). Thus, it appears that microglia play a vital role in the inflammatory cascade following SCI that can be either pro inflammatory or anti-inflammatory.

1.1.5.5 Astrocytes and Glial Scarring:
Following injury, the blood spinal cord barrier is disrupted (for up to 2 weeks) as a result of both mechanical forces and the influx of inflammatory mediators (Renault-Mihara et al., 2008). The activation and recruitment of astrocytes to the injury leads to reactive astrogliosis, and glial scarring where astrocytes hypertrophy occurs with the production of intermediate filaments as glial fibrillary acidic protein (GFAP) and vimentin (Wilhelmsson et al., 2004), extracellular molecules such as, chondroitin sulfate proteoglycan (CSPG) which inhibit axonal regeneration (Schwab et al., 2006). In this manner, Astrocytes build a wall around the injury site preventing axonal regrowth as shown in figure 5 (Anwar et al., 2016).
Central cavitation takes place days to weeks after the primary SCI (Oyinbo, 2011). It is manifested by an expansion of the SCI lesion size leading to the formation of a cavity much larger size than the initial scar (Fehlings and Nguyen 2010).

Reactive astrocytes role is rather confusing. On one hand, they are implicated in tissue integrity preservation (Faulkner et al., 2004), regulation of excess neurotransmitters production and in the release of extracellular matrix (Von Boxberg et al., 2006). While, On the other hand, reduction of reactive astrogliosis by inactivation of CSPG (Menet et al., 2003) or blocking Calcium channels (Lee et al., 2000), decreases the obstruction of the glial scar against axonal sprouting and regrowth (Von Boxberg et al., 2006).

Wide arrays of genes are upregulated upon reactive astrogliosis (Zamanian et al., 2012), e.g. intermediate filament proteins as GFAP, nestin, vimentin, Cytokine, and s100 (Zamanian et al., 2012). These genes are shown to form a complex enhancing damaged membranes repair (Rezvanpour et al., 2011).

1.1.5.6 Inhibitory Molecules:
Two main groups of molecules inhibit axonal regeneration; the first is the proteoglycans. These are composed of a protein core linked through four sugar moieties to a glycosaminoglycan (GAG) chain which contains repeated disaccharide units (Johnson-Green et al., 1991). These molecules have demonstrated an inhibitory profile following CNS injury. They are mainly expressed by astrocytes, meningeal cells and oligodendrocyte precursors (Fawcett and Asher, 1999). They are composed of four main groups; Dermatan sulphate proteoglycans (DSPGs), Heparan Sulphate proteoglycans (HSPGs), Keratan sulphate proteoglycans (KSPGs) and Chondroitin sulphate proteoglycans (CSPGs) (Johnson-Green et al., 1991). CSPGs are the most studied group and it includes: Brevican, Aggrecan, Neural/Glial antigen 2 (NG2), Versican, Neurocan and Phosphacan.
On one hand, these molecules are important for pathfinding and guidance (Fukuda et al., 1997), but on the other hand, CSPGs rich areas can inhibit axonal growth (Faissner and Steindler, 1995).

The second group is the myelin associated molecules. Nogo A is one example of this group. It is a membrane protein which is expressed by oligodendrocytes and some neurons, it causes growth cone collapse and growth inhibition, when it binds to its specific receptor named NgR1 in the neuron's membrane (Schwab, 2004). Another molecule in this group is the myelin associated glycoprotein (MAG) which is stored in Myelin that surrounds the CNS axons and is also originally produced by oligodendrocytes (Silva et al., 2014). It is regarded as one of the strongest inhibitors of white matter regeneration (Silva et al., 2014).

1.1.5.7 Apoptotic cell death:
Cell death after the mechanical trauma to the spinal cord is either due to necrosis which follows inflammation and cell membrane rupture (Oyinbo 2011), or due to apoptosis (Johnson et al., 2005), which begins within hours following SCI (Oyinbo 2011). The key players in apoptosis are caspases (Green 1998). Caspase 8 and 9 are the initiator caspases (Adjan et al., 2007), leading to further activation of caspase 3 (Wu et al., 2003). This in turn cleaves several downstream substrates that are crucial for apoptosis (Zhang et al., 2012). TNF-α acts as a mediator of apoptosis, through its death domain on its surface receptor TNFR1 (Zhang et al., 2012). However, there are conflicts regarding its capacity as being both pro and anti-apoptotic (Genovese et al., 2009).

1.1.5.8 Demyelinating Surviving Axons:
Oligodendrocytes cell death by excitotoxicity, apoptosis, inflammatory cytokines and free radicals can cause demyelination of the axons which survived the initial trauma (Oyinbo 2011) as shown in figure 5. Demyelination is evident in the subacute and chronic SCI phases (Guest et al. 2005) and it is due to oligodendrocytes loss in white matter rostrally and caudally weeks post SCI (Grossman et al. 2001).
1.1.6 Oxidative damage in SCI:
The spinal cord and CNS in general has high lipid content (in Myelin) and high oxygen need, rendering it easily susceptible to cell damage by lipid peroxidation (Tian et al., 2004). Following SCI, ROS overwhelm the antioxidant buffering mechanisms causing lipid peroxidation, cell lysis, and organelle damage and calcium imbalance as shown in figure 6. Thus antioxidant therapies such as high dose methyl prednisolone (MP) are currently the only useful medical intervention to enhance neuroprotection in SCI patients (Shan et al., 2010).

Free radicals cause a chain reaction which starts by absorbing an electron from a lipid molecule, eventually leading to membrane lysis, necrotic cell death and mitochondrial dysfunction (Sullivan et al., 2007), with an increase in intracellular calcium levels activating proteases and consequently breaking down cytoskeletal proteins as shown in figure 7 (Xiong et al., 2007).
Figure 5: Key players in SCI and glial scar formation. Demyelination, necrosis and axonal destruction takes place along with recruitment of microglia and astrocytes, leukocytes (neutrophils, macrophages, lymphocytes, natural killer cells). Reproduced from Anwar et al., 2016 with Permission.

These lipid peroxidation end products can be inactivated by a number of enzymes as glutathione-s-transferase, aldehyde reductase and aldehyde dehydrogenase (Ayala et al., 2014). ROS are also inactivated by other antioxidant defense systems such as glutathione peroxidase, catalase and superoxide dismutase, and other antioxidant mechanisms as vitamins A, E, and C, flavonoids and carotenoids as well (Bains and Hall, 2012).
Figure 6: Damage caused by oxidative stress following SCI. Oxidative stress causing neuronal death through DNA damage, mitochondrial dysfunction, protein aggregation and apoptosis. This damage can be prevented by phytodrugs. Reproduced from Pérez-Hernández et al., 2016 with Permission.
Figure 7: Oxidative damage cascade leading to Calcium influx causes rapid calpain activation. Excess calcium is taken up by mitochondria elevating mitochondrial nitric oxide synthase (NOS) which compete for O2 radical forming peroxynitrite (PON). PON derived radicals partially inhibit calpain initially but then, sustained oxidative stress potentiate cellular damage outweighs the initial calpain inhibition causing further cytoskeletal degradation. Reproduced from Xiong et al., 2007 with permission.

1.1.7 Role of TNF alpha following SCI:
Tumor necrosis factor alpha (TNF-α) is one of the most well characterized cytokines. However, to date there is no obvious agreement or consensus on its role in acute CNS injury (Oyinbo, 2011). Its levels are elevated following SCI reaching its peak within one hour after primary injury (Dinomias et al., 2009). Most studies suggest that its production at the site of injury exacerbates tissue damage in SCI (Pan et al. 2003, Paterniti et al. 2009). TNF α potentiates Wallerian degeneration of axons and activates recruitment of
macrophages and Schwann cells at the injury site (Sebastian and Schroeter 2003). It also increases the level of hyperalgesia and central sensitization through stimulation of synaptic transmission (Kawasaki et al., 2008).

Yakovlev and Faden showed that TNF-α mRNA levels were elevated 30 minutes following SCI at the injury site and that the extent of injury was proportional to the level of TNF-α message (Yakovlev and Faden, 1994). Paterniti et al. demonstrated that elevation of TNF α in a spinal cord trauma model which was accompanied by an elevation in myeloperoxidase activity (MPO) – a marker for polymorphonuclear leukocyte accumulation 24 hours post injury (Paterniti et al. 2009).

1.8 Current Interventions in Spinal Cord Injury:
As the primary injury to the spinal cord cannot be prevented it can only be minimized through surgical intervention by means of surgical decompression or stabilization of the vertebrae. Currently there are no generalized standards of care regarding decompression surgery regarding indications and timing of interventions (Li & Walker et al., 2014). Many questions remain unanswered in this regards, as the optimal timing of intervention to gain neuroprotective effect or even if the intervention itself is recommended or not (Silva et al., 2014).

Many strategies have been developed to minimize the extent of secondary injury, and enhance the therapeutic outcomes. Methylprednisolone is the most widely prescribed pharmacological agent for SCI yet it is the most controversial. It has many advantages as its ability to inhibit lipid peroxidation, limit inflammatory response, sustain the blood-spinal cord barrier and enhances blood flow (Tator, 1998). Other approaches include altering neuro-inflammation by means of immune-modulators such as minocycline (Schwartz and Yoles 2006) or promoting M2 macrophage phenotype through administration of granulocyte colony stimulating factor (G-CSF) within 72h following SCI (Guo et al., 2013).
Other mechanisms were adopted to enhance regeneration and myelination following SCI via creating a more suitable environment for axonal regeneration (Thuret et al. 2006). Nogo-A suppression (Freund et al., 2006), stem cells (Webb et al., 2010), and human embryonic stem cells (hESC) derived oligodendrocyte progenitor cells (OPCs) transplantation (Keirstead et al., 2005). Using Neurotrophins (Sharma, 2007), Autologous Activated Macrophages (Bomstein et al., 2003), reducing neuro-inflammation (Bracken 2012, Das et al., 2011), preventing free radical damage (Bains and Hall, 2012), and excitotoxicity (Mazzone and Nistri, 2011), and improving blood flow (Ritz et al., 2010), as well as targeting the local immune response (Varma et al., 2013), have all been used following injury with great variability in improving motor function, decreasing lesion volume, enhancing axonal regeneration and myelination.

Very few studies were advanced to clinical trial stage, e.g. the Sygen Multi Center Acute SCI study, which used variable doses (200, 300, and 600 mg IV) of monosialotetrahexosylganglioside GM-1 for 56 days. This study did not show a significant clinical efficacy of GM-1 but proposed a trend in favor of (GM-1) regarding American Spinal Injury Association (ASIA) motor, pin prick and light touch scores, contribution to neurological recovery and enhancing bowel / bladder function and improving sacral sensation as well (Chinnock and Roberts, 2005; Geisler et al., 2001). Rho antagonist (BIOAXONE BIOSCIENCES INC, Cethrin®) is another example for a clinically tested drug in SCI that was found to enhance neuronal growth (Dergham et al., 2002). Thus more research and clinical trials are needed to validate the benefit and efficacy of current proposed treatments for SCI.

1.1.9 SCI Models
Models of SCI aim at simulating human SCI injuries as much as possible (Cheriyan et al., 2014). The following are some examples of these models shown in figure 8.
Compression and Contusion models: It induces acute transient SCI via weight drop apparatus, air gun devices, or electromagnetic impactors (Cheriyan et al., 2014). This
model is clinically relevant as most of human SCI are caused by rapid movements, bone fractures and consequent impact of bone fragments (Allen, 1911). It is characterized by rostral and caudal spread of tissue damage from lesion epicenter, disruption of white matter axons and cell death of grey matter neurons, intra-parenchymal hemorrhage, glial activation, macrophage recruitment and oligodendrocyte apoptosis (Anwar et al., 2016). In some cases contusion is followed by sustained compression of the spinal cord. Experimentally this is simulated through clip compression (Rivlin and Tator 1978), balloon compression (Vanický et al., 2001) or calibrated forceps compression (Plemel et al., 2008). It induces vascular derangements, ischemia and hemorrhagic necrosis (Anwar et al., 2016).

**Distraction model:** it entails stretching of the spinal cord to induce tension forces that mechanically tear the spinal tissues leading to SCI (Silberstein and McLean 1994).

**Dislocation:** Involves displacement of the vertebra causing trauma to the cord which leads to astrocytic activation, vascular impairment in grey matter and apoptosis (Anwar et al., 2016). No surgical exposure is needed for this type of SCI model; however, more research is required to validate its reproducibility (Cheriyan et al., 2014).

**Transection models:** Complete or partial transaction is not regarded as clinically pertinent models; however, they have been vastly used to study neuronal outcomes following injury (Chriyan et al., 2014). These models are useful in studying axonal degeneration and regeneration following SCI (Anwar et al., 2016).

**Chemical model:** Such as injection of phopholipase A2 to induce inflammation (Liu et al., 2006), or inducing oxidative damage through injection of hydroxyl peroxynitrite into the rat’s gray matter. (Bao et al., 2003)

**Ischemia reperfusion injury:** induced by aortic cross clamping causing neuronal and vascular damage, it is regarded as a non-reproducible model (Anwar et al., 2016).
1.2 Curcumin
Phytodrugs represent an important approach in CNS disorders due to their neuroprotective abilities. Many plants’ raw extracts or their pure isolated compounds have demonstrated effective neuroprotective properties in neurodegenerative diseases (Pérez-Hernández et al., 2016). Silymarin administration (400mg/kg/day for 3 days) increased glutathione and superoxide dismutase (SOD) levels in aged rats brains (Galhardi et al., 2009). While daily doses (15 mg/kg) of Vincamine in rats reduced iron levels by 50% thus reducing oxidative stress resulting from iron deposition in neurodegenerative diseases (Fayed, 2010).

Resveratrol a Mediterranean traditional phyto phenol present in grapes has also shown neuroprotective traits. In focal cerebral ischemia model resveratrol significantly minimized infarction size suggesting a potent neuroprotective effect due to its antioxidant, anti-platelet and vasodilating effect in focal cerebral ischemia model (Huang et al., 2001).
Gingerol, a compound of Ginger which is extracted from the root of *Zingiber officinale* was shown to inhibit LPS induced cyclooxygenase 2 (COX-2) level of expression in U937 cells subjected to lipopolysaccharides, thus suggesting a role as an anti-inflammatory inhibiting prostaglandin E2 (PGE2) (Lantz et al., 2007).

Curcumin was first isolated by Vogel and Pelletier in 1815 in an impure form, later on both its chemical structure and synthesis were confirmed in 1910 and 1913 by Lampe et al. The first study for its use in human disease was published in 1937 (Oppenheimer, 1937). Throughout the last 60 years, over 3000 studies have described its variable biological activities as an antioxidant, antifungal, antibacterial anti-proliferative, pro-apoptotic, antiviral and other activities, consequently exhibiting medicinal benefit in neurodegenerative diseases, cardiovascular diseases, diabetes, psoriasis and lung fibrosis and other diseases (Aggarwal and Harikumar, 2009). This pleiotropic nature of curcumin has been attributed to its ability to interact with multiple targets including inflammatory cytokines, growth factors, transcription factors, apoptosis related proteins, adhesion molecules, kinases and others (Zhou et al., 2011).

Curcumin is a bis α, β unsaturated β diketone. Curcumin is one of turmeric constituents. Turmeric powder consists of 4% curcuminoids, 70% carbohydrates, 6% proteins, 5% resins, 4% fat and 10% moisture as shown in figure 9. Curcumin has also been used over the past centuries in Ayurveda or Ayurvedic medicine where it showed a great therapeutic efficacy in different respiratory conditions, liver disorders, diabetic wounds, anorexia and rheumatism (Araujo and Leon 2001). In Chinese medicine it has been used for abdominal pain (Aggarwal, Takada & Oommen, 2004) while in Hindu medicine it was applied for swellings and sprains (Araujo and Leon 2001). In the Orient it was mainly used as an anti-inflammatory (Aggarwal et al. 2003).

Many other therapeutic effects of Curcumin have been documented, such as its role as an antioxidant (Liu et al., 2013; CONEAC et al., 2017), anti-inflammatory (Machova et al., 2015; Gokce et al., 2016), hepatoprotective (Palipoch et al., 2014), anticarcinogenic (Rao
et al. 1995), hypoglycemic (Chuensamarn et al., 2012), renoprotective (Ueki et al., 2013), in cardiovascular diseases (Ahuja et al., 2011) and anti-arthritic (Dcodhar et al. 2013).

Studies show that Curcumin can promote neurogenesis in the adult hippocampus (Kim et al. 2008), inhibition of astrocyte expression, and preventing death of hippocampal cells by Kainic acid (Shin et al. 2007). Curcumin is characterized by a good safety profile demonstrated in animal (Qureshi et al. 1992) or human studies (Lao et al. 2006).

![Figure 9: Composition of turmeric powder. Curcuminoids constitute about 5% of turmeric components while the major constituent of turmeric is carbohydrates (70%) Reproduced from Farooqui 2013 with Permission.](image)

### 1.2.1 Curcumin Structure:

The main constituents of commercially available curcumin are; Curcumin I (77%), Curcumin II called demethoxycurcumin (17%) and Curcumin III called bisdemethoxycurcumin (3%) as shown in figure 10, where these curcuminoids collectively are found in 3-5% of Turmeric (Goel et al. 2008). This yellow complex has multiple names, e.g. yellow ginger, Indian saffron, yellow root or kacha haldi.

Curcumin powder is soluble in organic solvents as dimethyl sulfoxide, ethanol and acetone but insoluble in water or ether, it has a melting point of 183°C, a molecular weight of 368.37 g/mol and a molecular formula of C_{21}H_{20}O_{6} (Goel et al. 2008).
Curcumin is more stable at acidic PH while unstable at basic and neutral PH where it is degraded to ferulic acid and feruloylmethane. If placed in phosphate buffer systems of PH 7.2, it is degraded rapidly within 30 minutes (Wang et al., 1997).

Figure 10: Different chemical structures of Curcuminoids. Curcumin I compose about (77%) of the total curcuminoids, Curcumin II (demethoxycurcumin) and Curcumin III (bisdemethoxycurcumin) are present in smaller ratios. Reproduced from Farooqui 2013 with permission.

Curcumin degradation is slow at PH 1-6 which mimics the stomach PH. While one of the major Curcumin metabolites called tetrahydrocurcumin is more stable at neutral or basic PH (Pan et al. 1999), keeping its antioxidant abilities (Somparn et al., 2007).

Curcumin has shown to exhibit poor bioavailability for three main reasons: its poor absorption, rapid metabolism in the liver and rapid systemic elimination due to its short half-life (Bisht and Maitra 2009). Curcumin has shown to be highly tolerable. Phase 1 and 2 clinical trials of Curcumin have been carried out on patients with advanced colorectal cancer for 4 months at variable doses (500, to 12000 mg/day) showed no toxicity (Cheng et al. 2001). It showed that the serum concentration peaks at 1-2 hours following oral intake of Curcumin, followed by gradual decline within 12 hours (Jiao et al., 2009).
Curcumin metabolites do not have the same power or activity as the parent molecule where it has been reported that the reduced or conjugated forms of Curcumin do not have the same inhibitory effect on inflammatory enzymes expressed in human colon cells culture (Ireson et al. 2001).

To make the best use of its therapeutic properties, many approaches have been proposed to enhance Curcumin’s bioavailability, e.g. using an adjuvant as Piperine which interferes with the process of glucuronidation in the liver and intestinal metabolism, and complexing Curcumin with phospholipids. (Indena, Meriva®) - A phosphatidylcholine-Curcumin complex- showed plasma concentration and area under the curve (AUC) were 5 times higher than free Curcumin (Marczylo et al., 2007), hence showing an enhanced bioavailability pattern.

Also, preparing Curcumin liposomes and nanoparticles have been reported to remarkably increase free Curcumin’s bioavailability (Gota et al., 2010). Where mean peak plasma concentration of 650 mg of solid lipid Curcumin nanoparticle (SLCP) was 22.43 ng/ml while the same quantity of the unformulated Curcuminoid extract (95%) was undetectable; however, the study reported remarkable inter-individual variability in the two tested groups proposing complex absorption kinetics (Gota et al., 2010).

1.2.2 Curcumin as an Antioxidant and Anti-Inflammatory:

Curcumin is a highly pleiotropic molecule that affects variable molecular targets involved in neuro-inflammation, oxidative stress and neuroplasticity (Farooqui 2010). Curcumin possesses anti-inflammatory and anti-oxidative powers by downregulating enzymes as lipoxygenase (LOX), COX2 and inducible nitric oxide synthase (iNOS), these enzymes enhance the generation of several inflammatory mediators as leukotrienes, cytokines and TNF α (Farooqui et al. 2013).
Curcumin was shown to protect biological membranes including neural membranes against peroxidative damage, due to its ability to scavenge free radicals resulting from peroxidation (Menon and Sudheer, 2007). This unique ability is due to the presence of functional groups including carbon-carbon double bonds, β diketo group and phenyl rings to which hydroxyl and methoxy groups are attached (Menon and Sudheer, 2007; Indira Priyadarsini et al., 2003). This remarkable antioxidant capability is comparable to Vitamin E (Nimse et al., 2015)

A study was performed to evaluate the antioxidant potential of Curcumin in rat SCI model demonstrated that SOD level was higher in the Curcumin group compared with MP group. Malondialdehyde (MDA) exhibited the lowest levels in Curcumin group. It was concluded that Curcumin exhibits an antioxidant capacity against oxidative damage following SCI (Kavakli et al., 2011)

Neuro-inflammation is a natural defense mechanism aiming at isolating the injured tissue from the healthy tissue; unfortunately it might cause the destruction of the injured and healthy tissues (Correale and villa 2004). Upon CNS injury, the main mediators of inflammation are astrocytes and microglial cells. Upon injury, these cells enhance the rapid release of a number of cytokines/ chemokines which degrade the neural membrane, help release arachidonic acid and lyso-phosphatidylcholine (Farooqui 2013). Arachidonic acid is consequently oxidized to the pro-inflammatory eicosanoids as prostaglandins, leukotrienes, and thromboxanes by the help of COX and LOX. On the other hand, lysophosphatidylcholine enhances the synthesis of the pro-inflammatory platelet activating factor. Thus this cascade of events intensifies an aggressive neuro-inflammatory process (Farooqui 2013).

Inflammation is finally terminated by a process of resolution through the release of anti-inflammatory mediators as IL-10, resolvins, protectins and lipoxins (Farooqui 2010) and
this process is a turning-off mechanism held by neural cells for the purpose of limiting tissue injury (Farooqui 2013).

Curcumin has displayed a remarkable anti-inflammatory potential. It decreases linoleic acid conversion to arachidonic acid (Farooqui 2013). It also blocks the ability of macrophages to uptake arachidonic acid therefore limiting its ability for eicosanoids production. Curcumin can also inhibit cyclooxygenases and lipoxygenases and thus inhibit prostaglandins and leukotrienes synthesis (Rajasekaran, 2011).

Previous studies also demonstrated that dietary Curcumin decreased phospholipase A2, phospholipase C and prostaglandin E2. Moreover products of the lipoxygenase and cyclooxygenase as prostaglandin E2 and 15 (S) hydroxyeicosatetraenoic acid from arachidonic acid was reduced in colonic mucosa and tumors in Curcumin fed animals versus control group (Rao et al., 1995).

Curcumin also showed the ability to decrease COX at the transcriptional level. A study by the American Association for cancer research (AACR) on human colon cancer cells concluded that Curcumin reduced COX-2 expression on the mRNA level in a time and dose dependent manner and inhibited cell growth as well (Goel et al. 2001).

1.2.3 Molecular Targets of Curcumin:

Curcumin has proved to be a highly pleiotropic molecule that can interact with multiple molecular targets. Curcumin can bind directly to these targets or act indirectly through modulation of their activities (Zhou et al., 2011).

From the proteins that act directly with Curcumin: DNA polymerase (Takeuchi et al. 2006), lipoxygenase (Skrzypczak et al. 2003) and tubulin (Gupta et al. 2006). It has been proven
that Curcumin can bind to different divalent metal ions as well as Fe, Cu, Mn and Zn (Ishihara and Sakagami 2005).

Curcumin might activate or attenuate the activity of different transcription factors depending on the particular target. For instance, a Curcumin analogue has shown an ability to suppress the activity of nuclear factor kappa B (NF-κB) by inhibiting its nuclear translocation in Mouse RAW 264.7 and its downstream inflammatory cytokines as tumor TNF α, Interleukin 1β (IL-1β) and interleukin 6 (IL-6) in macrophage cells and it was found to be even more potent than Curcumin (Olivera et al., 2012). Therefore, inhibiting the activation of many genes responsible for proliferation and cell survival as Cyclin D1, IL-6, COX-2 and matrix metallopeptidase (MMP 9), thus arresting the cell cycle, induces apoptosis and inhibiting cell proliferation (Aggarwal & Takada et al., 2004; Wang et al., 2015).

Curcumin loaded solid-lipid nanoparticles reduced the expression of pro-inflammatory cytokines in serum as TNF-α, IL-6 and IL-1β, increased expression of anti-inflammatory IL-10 cytokine (Wang et al., 2015).

In a study that aimed at revealing the key players behind the anti-inflammatory mechanism of Curcumin, Curcumin suppressed COX-2, inducible nitric oxide synthase (iNOS) mediators of inflammation. The study suggested that this effect was in part due to Janus Kinase/Signal Transducer and Activator of Transcription (JAK-STAT) inflammatory cascade inhibition in the brain microglial cells (Kim et al., 2003). Curcumin also suppressed (STAT-3) phosphorylation significantly and showed anti-proliferative effect assessed by Cyclin D1 in lung adenocarcinoma cells (Alexandrow et al., 2012).

Peroxisome proliferator- activated receptor gamma (PPAR-γ) is a member of a large and diverse group of proteins factors called the nuclear receptor family of transcription factors. It regulates different functions as inflammation, apoptosis, cell differentiation and lipid
metabolism (Farooqui 2013). Its main role is to downregulate macrophage, microglial activation and cytokine expression by inhibiting the activity of some transcription factors as NF-κB, activator protein 1 (AP-1) and STAT proteins (Ricote et al. 1998; Aggarawal et al., 2007). Xu et al have shown that Curcumin induces PPAR-γ gene expression in activated hepatic stellate cells (HSCs) (Xu et al., 2003). In another study, Curcumin activated PPAR-γ cells inhibited Moser cells (human colon cancer cell line) and could suppress cyclin D1 and epidermal growth factor receptor (EGFR) gene expression (Chen and Xu 2005).

The limited regenerative ability of the injured axons following damage or injury to the brain and spinal cord in the adult mammalian CNS is due to the inhibitory effect of various neurite outgrowth inhibitors at the injury site consequently impairing axonal regeneration and causing functional deficits (Kohta et al., 2009). Besides the myelin associated inhibitory molecules, extracellular matrix molecules also play a growth impeding role as well. Astrocytic activation and the release of extracellular matrix inhibitory molecules as CSPGs (Kohta et al., 2009), causes further impediment to axonal regeneration (Yamashita et al., 2005; Yiu and He, 2006).

Zheng and Chen concluded that Curcumin could enhance PPAR-γ activity in HSCs thus inducing apoptosis and suppressing cell proliferation and extracellular matrix (ECM) gene expression. They also concluded that PPAR-γ activation by Curcumin could interrupt transforming growth factor (TGF-β) pathway leading to the inhibition of connective tissue growth factor (CTGF) expression which is originally responsible for ECM overproduction (Zheng and Chen, 2006). Yuan et al. also demonstrated a correlation between TGF-β inhibition by Curcumin and decreasing the deposition of CSPG of the extracellular matrix, reducing glial scar formation and enhancing innervations in SCI (Yuan et al. 2015), this renders the environment more suitable for axonal regeneration following injury.
Nuclear factor E2 related factor-2 (Nrf2) is a transcription factor that controls basal levels of expression of a number of antioxidant genes as well as enhancing the expression and upregulation of a number of detoxifying, antioxidant and cytoprotective genes that reduce tissue injury (Element 2004). Moreover, it is proposed that Nuclear Factor E2 Related Factor-2/ Antioxidant Response Element (Nrf2/ ARE) activation within the astrocytes enhances neuroprotection to neurons through mediating the expression of genes essential for maintaining redox potential and scavenging of free radicals (Kraft et al. 2004).

Curcumin leads to increasing Nrf-2 level thus enhancing its binding to ARE and increasing the expression of heme – oxygenase 1 (HO-1) in both neural and non-neural cells through activating UDP-glucuronosyltransferase (UGT) which is responsible for detoxification of many toxins present in our daily diet where HO-1 plays a pivotal role in cytoprotection from toxins of either endogenous or exogenous origin thus enhancing neuroprotection (Hatcher et al. 2008; Scapagnini et al., 2011).

Growth factors together with their receptors play a crucial role in the normal differentiation and growth process. Any irregularities in these molecules function or expression can cause abnormalities in development which might lead to malignant transformation (Witsch et al., 2010). Curcumin can regulate the expression of these growth factors thus demonstrating anti-invasive, anti-proliferative and anti-angiogenic effects (Zhou et al., 2011).

The epidermal growth factor receptor (EGFR, ErbB, and Her 1 in humans) is an integral protein kinase of the plasma membrane that comprises multiple autophosphorylation sites (Scaltriti and Baselga 2006). It was recently demonstrated that EGFR inhibition blocked pro-inflammatory cytokine secretion from reactive astrocytes and inhibited reactive astrogliosis as well in a scratch wound model in vitro, and reduced neuronal loss and demyelination which was concurrent with improvements in hind limb motor function and in bladder function as well in SCI rat model, thus proposing that EGFR inhibition might be a promising potential after CNS injury (Li & Li et al., 2014). Thus, if Curcumin has an
inhibitory effect on EGFR, this might provide a new mechanism of how Curcumin can impede the undesirable effects of reactive astrogliosis and enhance axonal regeneration.

In human astroglioma cell lines, Curcumin strongly suppressed phorbol 12-myristate 13-acetate (PMA) induced phosphorylation of extracellular signal–regulated kinases (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen activated protein Kinase (MAPK) thus suppressing MMP-9 enzymatic activity, mRNA and protein level as well as shown in figure 11 (Woo et al., 2005). This suggests that Curcumin can suppress AP-1 and NF-κB through inhibiting MAPKs pathway consequently adding to Curcumin’s potent anti-cancer and anti-inflammatory effects (Zhou et al. 2011).

![Figure 11: Curcumin effect on MAPK signaling pathway in human astroglioma cells. The figure shows MMP-9 signaling pathways in PMA treated U87MG cells. Curcumin inhibits (PMA) induced phosphorylation of ERK, JNK and p38 MAP kinase thus suppressing (MMP-9) enzymatic activity, mRNA and protein level, Reproduced from Woo et al., 2005 with permission.](image)

Pro-inflammatory cytokines as TNF-α, IL-1β and IL-6 are molecules produced excessively after severe injury or infection and they participate significantly in the development of systemic and local inflammation which might result in organ failure (Munford and Pugin 2001). The gene and protein cytokine expression is tightly regulated in the producing cells,
and one of the critical steps in this regulation is gene transcription, thus manipulating and regulating some of the transcription factors as NF-κB is a suggested strategy in controlling the inflammatory response (Baeuerle and Henkel 1994). Several research groups have demonstrated the ability of Curcumin to act as a potent anti-inflammatory by modulation of various inflammatory cytokines production (Chen et al., 2008). Both in vitro and in vivo studies have shown that Curcumin has a profound inhibitory effect on TNF-α production (Zhou et al., 2011). Curcumin prevented the release of pro-inflammatory cytokines, PGE2 and nitric oxide (NO) in a dose dependent manner. Curcumin also attenuated inducible NO synthase and cyclooxygenase-2 expression on mRNA and protein levels (Park, 2007).

Another group of inflammatory cytokines are the interleukins which play a crucial role in the regulation of the inflammatory response and in the signaling pathways as NF-kB and STATs which are involved in angiogenesis and tumor invasion (Dinarello, 2006). In an experiment done on TNF-α treated cells, Curcumin could attenuate expression of TNF α induced interleukin 1β (IL-1β) and interleukin 6 (IL-6) by inhibiting activation of NF-kB and MAPKs pathway (JNK, p38, MAPK, ERK) (Cho et al., 2007). A summary of Curcumin various targets is highlighted in figure 12.

Curcumin is able to form chelates with transition metals due to the presence β unsaturated β-diketone moiety. Metal chelates of Curcumin with Cu²⁺, Fe²⁺, Mn²⁺, Pb³⁺ Have been reported (Gupta et al., 2011). A study showed that Curcumin mononuclear complex with copper has anti-oxidant properties superior to Curcumin. The complex protected the cells against superoxide dismutase and glutathione peroxidase initially induced by radiation (Kunwar et al.,2007).Manganese complexes with Curcumin and its derivatives showed a potential capacity of protecting brain lipids from peroxidation (Sumanont et al., 2004).

Curcumin was found to bind to DNA via guanine and adenine N7 (major groove) and thymine O2 (minor groove) and to the phosphate (PO20 backbone as well. Curcumin binding to RNA was through H-bond to uracil O2 besides guanine and adenine N7 binding
as well as to the phosphate backbone (Nafisi et al., 2009). Further investigation is needed to explore the outcomes of Curcumin DNA binding especially regarding its noticeable nuclear localization and presence in tumor cells (Gupta et al., 2011).

Figure 12: Molecular targets of Curcumin. Curcumin can either upregulate or downregulate various molecular targets as Nrf2 and NF-kB respectively inducing a number of biological effects as antioxidant and anti-inflammatory. It also targets a number of cytokines, enzymes and growth factors affecting several biological processes. Reproduced from Shen et al., 2013 with permission

The role of Curcumin with DNA and other molecules still looks confusing and definitely more investigation in this arena is a necessity to clarify exactly the risk benefit ratio of Curcumin as a therapeutic agent either alone or in combination.
STUDY OBJECTIVES

The aim of this study is to investigate the role of Curcumin on the inflammatory and oxidative components of the secondary injury cascade following primary SCI in a rat model. We specifically aimed at comparing the effect of local application of Curcumin directly on the injury site with that of Dietary Curcumin supplement, by comparing the change in oxidative stress and inflammatory markers before and after Curcumin treatment.
CHAPTER 2: Materials and Methods

2.1 Establishment of SCI model:
A randomized controlled animal study using Female Sprague Dawley rats weighing approximately 150gm-200 gm were purchased from Ain Shams University, Cairo. The animals were allowed free access to food and water before surgical procedures. All experiments were performed according to the NIH (PHS Policy on Humane Care and Use of Laboratory Animals, 2015 (US Department of Health and Human Services 2015) and international guiding principles for biomedical research involving animals December 2012. All rats were anesthetized with ketamine (75 mg/kg) intraperitoneally. After shaving and cleaning the skin with Betadine, skin and muscle incision was made over the mid-dorsal section of the back. Laminectomy was performed at T9-T10 thoracic vertebral levels. The dura was incised longitudinally and spinal cord injury was performed by right side lateral hemisection using micro-iris scissors at T9-T10 level, Tail wagging reflex and hindlimb retraction proposed successful model establishment. Muscles and skin were then sutured in layers. All animals received analgesia (Voltaren 15 mg/kg) and local with systemic antibiotics (10 mg/kg) (Nair et al., 2016) were given post injury to prevent either surgical or urinary tract infections. Bladder evacuation was performed as needed during the experiment.

Two time points were investigated at our study and animals were grouped as follows:

- Control group n=15 normal rats and n=15 sham surgery rats (skin and muscle incision with no injury to the cord).
- Two Injury groups n=15 (at each time point) with lateral hemisection level at days 1 and 7 (SCI 1d and SCI 7d)
- Two local treatment groups n=15 (at each time point) with Curcumin (Curcuma longa extract, Herb Pharm, Williams, OR) 200 mg/kg single dose (Yuan et al., 2015) at the injury site immediately after injury (SCI+CUR 1d and SCI+CUR 7d)
• Dietary supplement group n=15 (SCI+ Diet 7d) where crude Curcumin (Imtinan, Egypt) was mixed with diet for one week before and one week after injury as 10% of daily feed.

At the time of analysis, an average of 4-6 rats were used for each marker where rats were sacrificed with IP ketamine overdose after 1 day or 7 days for sample collection, the spinal cord was exposed at the injury site where a segment including the injured area was removed and properly preserved.

2.2. Oxidative Stress Parameters:

2.2.1 Malondialdehyde test by Thiobarbituric Acid Assay

Malondialdehyde (MDA) is an end product of lipid peroxidation. The level of Malondialdehyde was determined using Thiobarbituric acid (TBA) assay (BIODIAGNOSTIC). TBA reacts with Malondialdehyde (MDA) at 95°C for 30 minutes forming a colored reactive product that can be measured. Spinal cord samples were homogenized in ice cold Phosphate buffered saline (PBS, pH 7.4) supplemented with protease cocktail inhibitor (Sigma-Aldrich). Samples were centrifuged at 10000 g for 15 minutes at 4°C. The supernatant was then removed to be used in the assay at -80°C. Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) at 95°C for 30 minutes forming a reactive product of a pink color that can be measured at 534 nm. Samples were incubated with TBA for 30 minutes in boiling water and absorbance was measured at 534 nm. Samples were run in duplicates. MDA is expressed as nmol/gram wet tissue.

2.2.2 Total Antioxidant Capacity:

The total antioxidant capacity in spinal cord samples (TAC) was determined using colorimetric assays (BIODIAGNOSTIC). This test is used as a measure of the ability of antioxidant systems in a sample to buffer increased reactive oxygen species (ROS).
The principle of the test is that exogenous H$_2$O$_2$ is added where a part of it is removed by the power of the antioxidants in the sample and the residual amount is determined in a colorimetric manner through an enzymatic reaction that converts 3,5-dichloro-2-hydroxybenzene sulphonate into a colored product.

Spinal cord tissue samples were homogenized in ice cold Phosphate buffered saline (PBS, pH 7.4) supplemented with protease cocktail inhibitor (Sigma-Aldrich). On performing the assay samples were centrifuged at 10000 g for 15 minutes at 4°C. The supernatant was then removed to be used in the assay at -80 °C. Samples were run in duplicates. TAC is expressed as mM/L. TAC and MDA assays were analyzed at Cairo University Research Park, Faculty of Agriculture, Cairo University using STAT LAB SZSL60-Spectrum.

2.3 Inflammatory Markers Testing by Enzyme Linked Immunosorbent Assay (ELISA):

Spinal cord samples (including the injury epicenter) were homogenized using ice cold PBS, pH7.4 supplemented with protease cocktail inhibitor (Sigma -Aldrich) and centrifuged at 10000 g for 15 minutes at 4°C. The supernatant was then removed to be evaluated for TNF-α enzyme linked immunosorbent (ELISA) assay kit (WKEA MED SUPPLIES CORP, New York, USA), and IL-6 ( R&D systems, R6000B ) according to the manufacturer’s guidelines.

Briefly, the microplate is coated with an antibody specific to TNF α or IL-6 Standard dilutions were prepared according to the manufacturer’s guidelines, samples were diluted 1:5 using calibrator diluent, wells were washed and conjugate ( HRP enzyme catalyzed conjugate) was added followed by incubation. Finally substrate solution was added and incubated followed by stop solution where OD readings were determined. All samples were run in duplicates. Finally, the concentrations of the samples were calculated from the constructed standard curve represented as Pg/ml.
2.4 Neurological evaluation
In our study, a neurological assessment using modified Tarlov Criteria (Shi et al., 2010) was performed. The criteria were as follows: 0 (no movement), 1 (minimal movement), 2 (good movement), 3 (stand and walk but cannot hop) and 4 (normal). A behavioral assessment using the grid walk (sensorimotor test) and the inclined plane test (motor test) was also conducted by 7 days treatment compared to simple injury group.

2.5 Histological Staining
Following anesthesia, rats were sacrificed and the spinal cord sections surrounding the injury epicenter were removed, dehydrated, embedded in paraffin and cryosectioned into 5 \( \mu \text{m} \) thick sections. Pathologic changes of the spinal cord were detected using hematoxylin and eosin staining. Histological examination of spinal cord sections for local treatment and dietary treatment groups to be completed.

2.6. Statistical Analysis:
All results were tabulated and statistical analysis was performed using IBM SPSS program (SPSS Inc., Chicago, IL, USA) , version 20, and all graphs were done using Microsoft Excel 2010. Quantitative data was represented as Mean ± Standard Deviation. One-way ANOVA test was used to compare means of quantitative variables. If ANOVA test was positive, Dunnett post Hoc test was used to identify the variable that made the significant change. Independent t-test was used to compare means of quantitative variables within SCI and SCI+CUR groups. All \( p \)-values are two-sided, and difference was considered statistically significant if \( p \leq 0.05 \) (95% confidence interval).
CHAPTER 3: RESULTS

3.1 Effect of Local and Dietary Curcumin on Oxidative Stress Markers in Spinal Cord Tissue

To study the effects of Curcumin on oxidative damage following SCI, we measured MDA and TAC levels in spinal cord tissue at 1 and 7 days post injury after local treatment (200 mg/kg) with Curcuma longa extract (Herb pharm, Williams, Oregon). For the dietary supplement with crude Curcumin MDA and TAC levels were measured only at 7 days post injury.

In this section results are demonstrated for:

- 1 day after SCI: Control group, Injury (SCI group) and Local treatment group (SCI+CUR).
- 7 days after SCI: Control group, Injury (SCI group), Local treatment group (SCI+CUR) and Dietary supplement group (SCI + Diet).

Groups will be referred to as Control, injury (SCI), local treatment (SCI+CUR) and Dietary supplement (SCI + Diet).

3.1.1 Local Curcumin Significantly Decreases MDA Levels 1 Day after SCI

Malondialdehyde (MDA) was measured in spinal cord tissue samples in all groups; control, injury at 1 and 7 days (SCI 1 day and 7 days), local treatment (200 mg/kg SCI + CUR 1 day and 7 days), and Dietary supplement group (SCI+ Diet) using Thiobarbituric Acid assay (TBA) assay and expressed as nmol/g wet tissue. As shown in figure 13, 1 day after SCI, MDA levels in SCI group were significantly elevated compared to control group (P < 0.01). Following local treatment (SCI+CUR), MDA levels were significantly lower when compared to SCI group p<0.05. Tables are represented in appendix.
3.1.2 Local Curcumin Significantly Decreases MDA levels 7 Days after SCI

At day 7 following local treatment (SCI+CUR), MDA levels were significantly lowered when compared to SCI p< 0.05. However, diet supplement (SCI +Diet) did not induce a significant reduction in MDA levels when compared to SCI group as shown in figure 14. Tables are represented in appendix.

Figure 13: MDA levels in spinal cord tissue 1 day after SCI. MDA levels for Control, SCI, SCI+CUR in homogenized spinal cord tissue. MDA represented as nmol/g wet tissue weight. n = (4-6), all samples were run in duplicate. Error bars are standard deviation (**p<0.01 vs. control group and # p<0.05 vs. SCI group). (One way ANOVA, Dunnett post hoc, Independent t-test).
Figure 14: MDA levels in spinal cord tissue 7 days after SCI. MDA levels measured for Control, SCI, SCI+CUR and SCI+Diet groups in homogenized spinal cord tissue. MDA represented as nmol/g wet tissue weight. n = (4-6), all samples were run in duplicate. Error bars are standard deviation (#p<0.05 vs SCI group). (One way ANOVA, Dunnett post hoc).
3.1.3 Total Antioxidant Capacity 1 Day after SCI and Local Curcumin Application
Total antioxidant capacity (TAC) was measured to detect the effect of Curcumin as an antioxidant following SCI. TAC was measured as mM/L of homogenized tissue samples. An elevation in TAC might be an indication of an antioxidant capacity exerted by Curcumin treatment. As shown in figure 17 at day 1 after SCI, TAC levels were significantly lowered in all injury and treatment groups compared to control p < 0.01 as shown in figure 15. After local treatment (SCI +CUR), no significant elevation in TAC levels was detected when compared with SCI group. Tables are represented in appendix.

3.1.4 Local Curcumin and Diet supplement significantly elevate TAC 7 Days after SCI.

TAC levels were significantly decreased in SCI + local treatment group (SCI+CUR) when compared to control group p<0.01 at 7 days after SCI. However, TAC levels were significantly elevated by local treatment regimen (SCI+CUR) p < 0.05 and by Dietary supplement group (SCI + Diet) p< 0.01 compared to SCI group as shown in figure 16. Moreover, TAC levels were significantly elevated by diet supplement when compared to local treatment regimen p<0.01 as sown in figure 16. Tables are represented in appendix.
Figure 15: TAC levels in spinal cord tissue 1 day after SCI. TAC levels measured for Control, SCI, SCI+CUR in homogenized spinal cord tissue. TAC represented as Mm/L. n = (4-6), all samples were run in duplicate. Error bars are standard deviation. (**p<0.01 vs. Control group. (One way ANOVA, Dunnett post hoc, Independent t-test)
Figure 16: TAC levels in spinal cord tissue 7 days after SCI. TAC levels measured for Control, SCI, SCI+CUR and SCI+Diet in homogenized spinal cord tissue. TAC represented as Mm/L. n = (4-6), all samples were run in duplicate. Error bars are standard deviation. (**p<0.01 vs.Control, ##p<0.01 vs. SCI group, #p<0.05 vs. SCI group, ɵ p<0.01 vs.SCI+CUR group). (One way ANOVA, Dunnett post hoc).
3.2. Effect of Curcumin on Inflammatory Cytokines Expression in Spinal Cord Tissue

To determine the effect of Curcumin on the inflammatory process following SCI, we measured TNF α and IL-6 levels in spinal cord tissue after local treatment

- TNF α levels were measured at 7 days post injury for both local treatment (SCI + CUR 200 mg/kg) with Curcuma longa extract (Herb pharm, Williams, Oregon) and dietary supplement regimen (SCI + Diet) (1 day TNF α values to be completed).
- IL-6 levels were measured for 1 day and 7 days local treatment (SCI +CUR 200 mg/kg) with Curcuma longa extract (IL-6 levels for Dietary supplement group to be completed).

3.2.1 Local Curcumin and Diet Supplement Significantly Decrease TNF α Expression Levels

TNF α levels were measured in rat spinal cord tissue in control, injury and treatment groups using Enzyme Linked immunosorbent Assay (ELISA). TNF α was measured as Pg/ml.

As shown in figure 17 at 7 days after SCI, TNF α levels were significantly elevated in SCI group p< 0.01 compared to control group. Upon treatment, TNF α levels were significantly lower in local treatment (SCI+CUR) and Diet supplement groups (SCI+Diet) compared to SCI group p<0.01. Tables are represented in appendix.
Figure 17: TNF α expression levels in spinal cord tissue 7 days after SCI by ELISA. TNF alpha levels for Control, SCI, SCI+CUR and SCI+Diet in homogenized spinal cord tissue represented as Pg/ml. n=(4-6), all samples were run in duplicate. Error bars are standard deviation. **p< 0.01 vs. control, ##p< 0.01 vs SCI. (One way ANOVA, Dunnett post hoc).
3.2.2. IL-6 Expression Levels 1 Day after SCI and Local Curcumin Application

IL-6 expression was measured in rat spinal cord homogenized tissue samples using ELISA. IL-6 is expressed as Pg/ml. At 1 day after SCI, IL-6 levels were significantly elevated in SCI and local Curcumin treatment (SCI+CUR) compared to control group p< 0.01 and p < 0.05 respectively. No significant lowering of IL-6 values was detected upon local treatment (SCI+CUR) when compared to SCI group at 1 day as shown in figure 18. Tables are represented in appendix.

3.2.3 Local Curcumin Significantly Decreases IL-6 Levels 7 Days after SCI

At 7 days, IL-6 levels were significantly elevated in SCI and local treatment group (SCI+CUR) compared to control group P<0.01. Upon local treatment, IL-6 levels were significantly lowered compared to SCI group P< 0.05 as shown in figure 19. Tables are represented in appendix.

Figure 18 : IL-6 Expression levels in spinal cord tissue 1 day after SCI measured by ELISA. IL-6 levels for Control, SCI, SCI+CUR in homogenized spinal cord tissue represented as Pg/ml. n=(3), all samples were run in duplicate.Error bars are standard deviation. (*p< 0.05, ** p<0.01 vs. Control group).(One way ANOVA, Dunnett post hoc, Independent t-test).
Figure 19: IL-6 expression level in spinal cord tissue 7 days after SCI measured by ELISA. IL-6 for Control, SCI, SCI+CUR and SCI+Diet in homogenized spinal cord tissue represented as Pg/ml. n=(3), all samples were run in duplicate. Error bars are standard deviation. (**p<0.01 vs. Control, # p< 0.05 vs SCI group). (One way ANOVA, Dunnett post hoc, Independent t-test).
3.3 Neurological Function Evaluation

In our study, behavioral assessment has shown that Curcumin treatment groups exhibit a trend towards improved functional activity in treatment groups relative to injury groups. However, our results didn’t evaluate the functional improvements on longer term, yet an improvement upon injury was noticed when treatment groups were compared to injury group 7 days after SCI.

Thus a complete behavioral assessment was not presented. However, this is a long term study and since our main goal is to compare between dietary regimen and local regimen we assume that motor function test starting at 7 days would reflect more reliable data especially regarding diet supplement regimen. Thus, in order to validate our data we intend to complete the neurological assessment applying the scoring system between different groups on longer term to obtain a more descriptive and accurate data on the effect of Curcumin treatment on neurological function.

3.4 Histology

Spinal cord sections were examined by hematoxylin and eosin staining. Normal spinal cord sections and spinal cord sections following SCI were examined. Following injury, the normal spinal cord structure was impaired where both grey matter and white matter were damaged; pronounced cavitation was detected accompanied by neuronal degeneration. Normal and injured spinal cord sections are shown in figures 20 and 21 respectively.
Figure 20: Hematoxylin and Eosin Staining Sections of Normal spinal cord. Spinal cord sections around injury were removed dehydrated, embedded in paraffin and cryosectioned into 5 μm thick sections. The normal tissue shows preserved tissue with the normal arrangement of the white matter, grey matter and neurons. (Magnification X40)
Figure 21: Hematoxylin and Eosin staining for spinal cord tissue following SCI. Spinal cord tissue was damaged with impairment of both white matter and grey matter with noticeable cavitation and neuronal degeneration. (Magnification X40)
Spinal cord injury is a debilitating condition characterized by a series of complex series of events, the primary injury causes tissue damage, axons demyelination, and cell membrane damage enhancing the initiation of secondary injury cascade (Kwon et al., 2004). Secondary damage involves multiple events such as ionic imbalance, release of free radicals, glutamate excitotoxicity, blood-spinal cord barrier damage and inflammation. These events are mediated and potentiated by glial cells, macrophages and T-lymphocytes (Anwar et al., 2016). It is well established that reduction of secondary tissue damage is a prime goal in treating spinal cord injury (Dumont et al., 2001).

The main focus of our study was to study the effects of Curcumin on two of the main hallmarks of secondary SCI; the inflammatory process and the oxidative damage. Our second goal was to find if dietary supplement of Curcumin would have a similar role to direct local application.

Curcumin is a naturally occurring nonsteroidal that manifests an array of beneficial pharmacological effects as anti-inflammatory, antioxidant, anticarcinogenic and antibacterial functions (Shishodia 2013). Thus detecting a potential role for Curcumin in spinal cord injury might add a potential tool in ameliorating the damaging cascade of events in SCI, as well as other CNS conditions thought to be caused by inflammation and oxidative damages. It has been proposed in several studies that curcumin administration enhances neuroprotection by decreasing inflammatory response, oxidative stress damage, glial scar formation and enhancing motor recovery as will be discussed below.

This study focuses on detecting the hypothesized role of Curcumin as a neuroprotective agent through its anti-inflammatory and an antioxidant effect. The main findings of this
study were as follows: 1) a significant increase in inflammatory and oxidative markers was noticed following injury, MDA, TNF α and IL-6 respectively. 2) Elevation in TAC levels following treatment specifically by 7 days. 3) Curcumin local treatment regimen showed an anti-inflammatory and antioxidant potential 4) Dietary curcumin supplement showed a beneficial effect; however, we conclude that both regimens need further modification.

Following SCI, a milieu is developed that shifts the balance pro-oxidant/ antioxidant towards oxidative stress (Bains and Hall, 2012). It results from failure of free radicals scavenging capacity. Free radicals cause lipid peroxidation and protein damage within spinal neurons, glia and other cells (Fatima et al., 2015). In SCI rat models, Malondialdehyde (MDA) elevation has been documented following injury (Varija et al., 2009). MDA is an aldehyde end product of lipid peroxidation in SCI and is regarded as an evidence of oxidative stress in rat SCI models (Liu et al., 2013, Kim et al., 2014).

In the present study Curcumin showed a noticeable antioxidant effect; however, varied depending on the treatment regimen applied. After injury, MDA levels were significantly elevated by 1 day compared to the control group, this is consistent with other studies which show that ROS generation in rat model is characterized by an elevation in MDA accompanied with reduction of antioxidant markers as superoxide dismutase (Paterniti et al., 2009; Liu et al., 2013; Guo et al., 2014). Xu et al. showed that 6 h after spinal cord compression injury, MDA levels were elevated in a modest; however, a significant manner, this elevation was also detected by immunohistochemical staining in motor neurons (Xu et al., 2005)

We noticed that in Curcumin local treatment groups (SCI +CUR 200 mg/kg), MDA levels were significantly lowered compared to injury groups either at 1 day or 7 days. This is consistent with other studies where Kavakli et al. reported a decrease in MDA level 24 h in serum post Curcumin treatment in a weight drop SCI model where Curcumin was given
200 mg/kg orally (Kavakli et al., 2011). Sanli et al. reported similar findings following Curcumin administration 300 mg/kg intraperitoneally (Sanli et al., 2011).

At 7 days we noticed a significant decline in MDA levels following local treatment compared to injury and control groups. This is not consistent with the findings of Kim and colleagues, who report a significant elevation in MDA level by 1 week that only lowered significantly at 2 weeks when compared to vehicle group (Kim et al., 2014). However, their findings were based on plasma levels and not in cord tissue as our current study. Another possibility is the difference in the model used where Kim et al used an aneurysm clip compression SCI model, while we used a hemisection model.

Curcumin Dietary supplement given 7 days before and 7 days after injury, did not affect MDA levels significantly when compared to 7 days injury group. We assume that the dietary regimen that we applied was not capable enough of affecting MDA values post injury. Moreover, Dietary supplement with crude Curcumin was introduced to the animals ad libitum, thus a variation between the amounts of Curcumin between animals might have affected the overall effect. Although our goal was to test the potential neuroprotective effects of Curcumin when used as a dietary supplement, we recommend to avoid such variability by introducing Curcumin in a consistent and reproducible manner by gavage as previously described (Reeta et al., 2009; CONEAC et al., 2017).

Total antioxidant capacity (TAC) represents the synergistic effect between different antioxidants (Valkonen and Kunsi, 1997). At 1 and 7 days, we noticed a significant decrease in TAC values in all injury and treatment groups when compared to control group except in the dietary supplement group where higher TAC values were detected. However, when comparing to injury group 7 days after SCI, both local treatment (200 mg/kg) and Dietary supplement induced a significant elevation in TAC values compared to 7 days injury group. Moreover TAC levels in Diet supplement groups were more significantly elevated when compared to local treatment group.
We assume that this effect by the Curcumin dose we used (200 mg/kg) was masked at 1 day by the surge of oxidative damage that follows injury; however, by 7 days this surge has subsided due to the initial treatment and Curcumin treatment possibly had a delayed effect.

Al Rubaei and colleagues detected higher TAC and lower MDA levels in Curcumin treated groups when compared with H2O2 experimentally induced oxidative stress group in liver tissue homogenate (Al Rubaei et al., 2014). In a rat inflammation model, oral Curcumin in carboxy-methylcellulose (150 mg/kg), liver Total antioxidant capacity (TAC) was increased significantly in Curcumin treatment groups where samples were collected 15 min to 3h after Curcumin treatment, while serum TAC levels in Curcumin/carboxy-methylcellulose group and inflammation were lower than Curcumin only group (CONEAC et al., 2017).

In this context we have to highlight three main points. First, at 1 day local treatment the significant decrease in MDA was not accompanied by significant TAC elevation when compared to injury group. Second, at 7 days local treatment (200 mg/kg) we noticed that TAC was significantly elevated and MDA was significantly lowered as compared to 1 week injury group. Third, dietary Curcumin supplement elevated TAC significantly; however, MDA levels were not significantly lowered when compared to SCI group.

Regarding the first point, MDA lowering might be attributed to an increased activity of a certain antioxidant enzyme such as SOD or Catalase which were reported to be elevated by Curcumin treatment in other SCI models, (Cemil et al., 2010; Kavakli et al., 2011; Kim et al., 2014) . Hence, MDA might have been affected by a certain enzyme (s) rather than the total antioxidant capacity in the spinal cord tissue.

Following dietary Curcumin supplementation, although TAC levels were significantly elevated, MDA values were not significantly lowered compared to 1 week injury group.
We assume that in the dietary supplement group the elevation of TAC was still not enough to cause MDA lowering, or possibly this elevation in TAC levels was not enough to induce the expression of antioxidant enzymes that affects MDA levels. Another possibility is that crude forms of Curcumin have a mix of active ingredients that induce various biochemical effects, therefore we recommend using a pure form of Curcumin extract, or other Curcumin derivatives such as (CNB-001; 500 ppm) should be considered. This derivative is more stable and enhances neuroprotection as well (Liu et al., 2008; Wu et al., 2011).

A proposed mechanism of Curcumin is that it enhances the activation of nuclear factor erythroid 2-related factor (Nrf2). Elevation in the level of antioxidant enzymes is regulated through antioxidant response element (ARE) that is present in the regulatory region of genes of antioxidant enzymes (Nguyen et al., 2003). ARE is an enhancer of a number of phase II antioxidant enzyme genes as Hemeoxygenase, reduced nicotinamide adenine dinucleotide phosphate quinine oxidoreductase and glutamate cysteine ligase (Wilson, 1997). When Nrf2 dissociates from kelch-like ECH associated protein 1 (keap1), it is translocated to the nucleus and binds to ARE enhancing the transcription of downstream target genes (Motohashi and Yamamoto 2004).

A study by Jiang and colleagues was the first to report the effect of Curcumin on Nrf2 in astrocytes, demonstrating that Curcumin enhanced phase II enzymes induction in Nrf2 +/- astrocytes where this effect was not evident in Nrf2 +/- astrocytes suggesting that Curcumin enhances Nrf2 cytoprotective role in astrocytes. Moreover, Curcumin inhibited reactive oxygen species (ROS) in Nrf +/- Astrocytes and not in Nrf +/- Astrocytes. They propose that this effect can add a lot to Curcumin therapeutic value especially as it can be a part of human diet or daily supplement with low toxicity (Jiang et al., 2011).

Jin et al. also demonstrated that Curcumin (IP injections 100mg/kg) enhanced of Nrf2 activity in SCI vascular clip compression model reduced locomotor impairment, apoptosis and spinal cord edema (Jin et al., 2014).
Other markers may also be used to evaluate oxidative stress following SCI and treatment efficacy, such as superoxide dismutase (SOD) (Kavakli et al., 2011) and hemeoxygenase (HO-1) (Diaz-Ruiz., 2013). The main identified free radicals are: superoxides (O2°-), hydroxyl radical (° OH), hydrogen peroxide (H2O2), Peroxynitrite (ONOO -) and nitric oxide (°NO) (Yang et al., 2013) where mainly the superoxide (Liu et al., 1998) and hydroxyl radical (Bao and Liu, 2004) are the most damaging and play the main role within the other ROS following SCI. They are produced by a complex system of enzymes in both microglia and immune cells as: nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase and cyclooxygenase (Bains and Hall, 2012).

Levels of TNFα are elevated following SCI, reaching its peak within one hour after primary injury (Dinomais et al., 2009). It is released faster than other inflammatory cytokines (Feldman, 2008) and it activates several other cytokines and growth factors release as well as immune cells recruitment. This chief role made TNF α an attractive target in inflammatory conditions including SCI (Esposito and Cuzzocrea, 2011). Studies suggest a dual role of TNFα. For instance, it is suggested that it might exert deleterious effects in acute phase versus beneficial effect in chronic phase after SCI (Chi et al., 2010) or exerting anti-apoptotic effect through TNFR-NFκB signal transduction pathway (Kim et al., 2001).

TNF α is secreted by microglia and it stimulates glutamate release which in turn through metabotropic glutamate receptors enhances more TNFα release. Consequently, it acts on astrocytes inducing glutamate release and upregulating its levels consequently. Due to this excitatory elevation, calcium entry is elevated causing excitotoxicity and neuronal death, thus TNF α links neuro-inflammation and excitotoxic effects (Olmos and Lladó, 2014).

Initially TNFα is synthesized as transmembrane protein where its extracellular domain is cleaved by matrix metalloprotease TNFα converting enzyme into the soluble form where TNFR1 signaling activates a number of transduction pathways as: JNK, NF-κB, ERK and
p38 MAPK (Byun et al., 2012; Olmos and Lladó, 2014) which makes it somehow ambiguous to clearly define which of them impacts TNF α expression (Olmos and Lladó, 2014). For instance, a study reported that TNF α expression was prevented through inhibition of MEK/ERK pathway. They also showed that NF-κB binding to DNA which is initially activated by interferon gamma (IFN γ) is completely dependent on TNFα release via MEK/ERK pathway in BV-2 microglial cell line (Mir et al., 2009). NF-κB pathway regulates promoters of cytokines producing genes as TNFα, IL-6 and IL-1β (Hang et al., 2004). This cascade amplifies initial inflammation and aggravates consequences in SCI following the initial trauma (Ni et al., 2015).

The production of IL-6 is in part modulated by many factors as IL-1b and TNFα. IL-6 production was correlated with glial scar formation (Brunello et al., 2000). IL-6 knockout caused astrogliosis suppression (Klein et al., 1997). Besides, following SCI noticeable impedance of axonal growth was detected in mice with increased IL-6 signals (Lacroix et al., 2002).

In the present study, TNFα levels were measured at 7 days in all groups (control, SCI, SCI+ local treatment and SCI+ Dietary supplement). TNF α levels were significantly elevated at 7 days injury in SCI group compared to control group. Further analysis is planned to evaluate 1 day expression across different groups.

IL-6 expression was significantly elevated in all groups either SCI (1 day and 7 days) or SCI +local treatment 200 mg/kg at 1 day and 7 days when compared to control group. However, data regarding Dietary supplement is not represented for IL-6 and yet to be completed.

The present data is consistent with previous reports that demonstrated alteration in the levels of inflammatory cytokines following SCI. In an SCI clamp model, serum levels of TNFα, IL-6, IL-10 and IL-1a gradually increased at 24h reaching peak at 3 days. Pro-
inflammatory cytokine expression started lowering at day 7, although shown to remain significantly higher than control group, and stabilizing at day 28 (Geng et al., 2015). In an SCI weight drop contusion model, IL-6 receptor expression quantified by western blot was increased 8 fold within 12 hours following injury when compared to before injury (Okada et al., 2004). In an aneurysm clip compression model showed that inflammatory cytokines including TNFα, NF-κB and IL-1β increased at 1 hour after injury and peaking at 12 hours SCI (Yuan et al., 2015). Guo and colleagues also showed an elevation in TNFα and IL-6 mRNA levels at 2h and 6 h respectively following spinal cord contusion, as well as, a significant increase in the TNF α and IL-6 protein levels at 24 hours following SCI (Guo et al., 2014).

This documented variability in peak times may be due to differences in severity of injury between different SCI models. The first study to compare severity dependent expression of IL-1β, IL-6 and TNFα following SCI, used a graded SCI contusion model. On the gene level, IL-6 and TNFα mRNA levels were increased at 1, 3 h and peaked at 6 h post injury returning to baseline levels at 1 d flowing severe injury, while these levels were significantly lower in the mild injury group. On the protein level, the cytokines including TNFα and IL-6 showed a marked but transient increase following severe SCI while in the mild injury group no cytokines were detected by western blotting (Yang et al., 2005).

The effects of Curcumin have been well documented; Chen et al. demonstrated that Curcumin treatment reduced IL-1β mRNA expression in astrocyte culture with no change in TNFα expression, while in microglia pretreatment with Curcumin decreased the elevated IL-1β and TNFα significantly (Chen et al., 2015). Another study showed that following contusion SCI TNFα is localized in the cytoplasm of the anterior horn neurons of the spinal cord and that the number of TNFα positive cells in gray matter was significantly elevated 24 h and at 3 d post injury. TNFα expression was significantly elevated at 3d and 5d (Zhang et al., 2015).
In our study we detected changes in TNF α and IL-6 in spinal cord tissue samples as an indicator for the inflammatory process following SCI hemisection model. After Curcumin administration, TNFα levels were significantly decreased in SCI + local (200 mg/kg) and SCI + diet groups when compared to 7 days SCI group. Furthermore at 7 days, IL-6 levels were significantly decreased Curcumin local treatment when compared to SCI group; however, at 1 day local treatment did not show a significant decrease. This might be due the strong recruitment of inflammatory cytokines by immune cells that might mask Curcumin effect. Nonetheless, Curcumin significantly lowered IL-6 level by 7 days, this might be explained by a delayed effect of curcumin that was not manifested at 1 day.

Machova et al used a single local dose of Curcumin followed by daily IP administration of Curcumin daily for 28 days following SCI using a balloon compression model TNFα levels were only significantly decreased at days 1 and 14. They demonstrated a transient elevation of TNF α levels between day 3 and 14; however, they assumed that it might be due to activation of the immune response cells which when stabilized TNFα levels decreased. They also proposed that Curcumin dose might have not been effective (Machova et al., 2015). They also demonstrated that IL-6 levels were significantly elevated upon treatment with curcumin when compared to vehicle control at days 14 and 28 (Machova et al., 2015). It is worth mentioning that Machova et al. study used a different SCI model and a different Curcumin treatment regimen than the regimens we used in the present study.

Machova et al. 2015 also showed that following treatment, no alteration was found in IL-6 levels at days 1, 3, 7 and 10; however, its level was increased by days 14 and 28. They proposed that Curcumin might act more effectively in early stages or that its effect is for a distinct period of time, but their detection window was till 28 days so the early phase in their study was 7 days which is regarded as the delayed phase in our current study. However, we believe that further investigation is needed to validate IL 6 changes at different time points.
Yuan et al. demonstrated that TNFα besides other inflammatory cytokines were elevated significantly at the gene and protein levels following SCI through aneurysm clip compression where Curcumin was given as 300 mg /kg once daily for 7 days. The elevation at the protein level was documented as soon as 1 hour following injury with a peak elevation at 12 hours post injury followed by a decline; however, they reported that the expression remained high in their therapeutic detection window which was 72 hours. In their study they correlated these high cytokine levels by a strong inflammatory response in acute SCI. Moreover, the authors suggested a correlation between the higher cytokine levels including TNFα, IL-1β and NF-κB in the simple injury group and a larger glial scar area aggravating secondary damage and tissue necrosis (Yuan et al., 2015).

Another study using vascular clip compression SCI model, showed that TNFα, IL-1β and IL-6 were elevated by 257.9%, 229.0% and 296.3% increase respectively compared to sham group. The authors suggested a mechanism behind this elevation, they showed that following SCI, an increased expression of toll like receptor 4 (TLR4) on the gene and protein level followed by upregulation of NF-kB was documented. In turn, this upregulation potentiates the activation of the inflammatory cytokines as TNFα which in turn activates NF-kB. Thus a positive feedback mechanism is initiated amplifying inflammation and exacerbating damage following SCI (Ni et al., 2015). Hence, they proposed that Curcumin therapeutic effect is due to modulation of TLR4/NF-κB inflammatory pathway and (Ni et al., 2015).

In a vascular clip compression model, Jin et al. showed that at 72 hours following injury, TNFα, IL-1β and IL-6 concentrations were significantly lower in the Curcumin treatment group when compared to the SCI group. Curcumin was administered in a similar dose to Ni et al. (intraperitoneally as 100 mg/kg in PBS including 1% DMSO). Jin and Colleagues demonstrated that curcumin administration up-regulated Nrf2 activity while NF-κB activity was down regulated. They proposed that Curcumin beneficial effect was due to enhancing
the activity of Nrf2, decreasing NF-κB and hence decreasing the activity of the downstream inflammatory cytokines (Jin et al., 2014).

Cytokines specific mechanism of action in neurodegeneration is considered complex and still unclear. They can either act directly on neurons or indirectly through affecting blood flow, glia or brain vasculature. Cytokines can act on many levels exerting multiple actions on different cells eventually causing excess calcium release, apoptosis, enhancing calcium uptake and free radical production (Allan and Rothwell, 2001).

In our study, neurological assessment has shown that Curcumin treatment groups exhibit a trend towards improved functional activity in treatment groups relative to injury groups. However, our results didn’t evaluate the functional improvements on longer term, yet an improvement upon injury was noticed when treatment groups were compared to injury group 7 days after SCI. A complete behavioral assessment was not presented as well. However, this is a long term study and since our main goal is to compare between dietary regimen and local regimen we assume that motor function test starting at 1 week would reflect more reliable data especially regarding diet supplement regimen. For instance another study performed the behavioral assessment at postoperative days 21 and 42 (Holly et al., 2012).

Several studies show functional improvement following Curcumin treatment in various SCI models. Liu et al. performed a neurological assessment test on ischemia/ reperfusion (I/R) SCI model 48 hours following reperfusion. They showed that after Curcumin treatment, although neurological score was lower than sham group yet it was higher than that of the I/R group significantly (Liu et al., 2013). Ni et al. used Basso, Beattie and Bresnahan (BBB) locomotor scale to assess forelimb-hindlimb coordination, hind limb movements and whole body movements as well. By 72 h following SC compression injury, BBB score was nearly zero; however, after Curcumin treatment 100 mg/kg IP the score was significantly increased (Ni et al., 2015). In a left hemisection SCI model, daily Curcumin
treatment (40 mg/kg) starting 1 day before surgery and continued until 6 days, showed that Curcumin treatment was accompanied by improved BBB score of the affected hind limb on days 3 and 7 (Lin et al., 2011). Yuan et al also reported a dose effect correlation where higher Curcumin concentrations caused more improvement of functional recovery on the BBB score when compared with methylprednisolone (MP) or with the simple injury group starting from week one till week 8 (Yuan et al., 2015).

Regarding the scope of our study, which is comparing the dietary regimen with local treatment regimen, a long term behavioral assessment should be considered to reliably evaluate if the regimens used would give a sustained motor function improvement or not. For instance Yuan et al. evaluated the effect of the given curcumin treatment from week 1 till week 8 (Yuan et al., 2015). Besides, Holly et al. compared different groups for behavioral assessment on postoperative day 21 and 42 where they were detecting the effect of docosahexaenoic acid-curcumin (DHA-Cur) diet on enhancing neuroprotection in cervical spondylotic myelopathy (CSM) rat model (Holly et al., 2012).

Potential limitations of our study could be the dietary regimen using crude turmeric which only contains 3-5 % Curcuminoids 60% of which is Curcumin (Gupta et al., 2013). Thus using a more stable and efficacious form as CNB-001 used by Wu et al. study might have represented clearer results. Another limitation was not looking at time points between 1 day and 7 days as some markers in our study have shown a change by 7 days thus their fluctuation pattern before 7 days should be investigated. Moreover, time points beyond 7 days should be considered as well for investigating the sustainability of Curcumin either biologically or regarding on motor function assessment following treatment.

In another study, Curcumin was applied locally then systemically throughout the experiment (Machova et al., 2015). We should consider daily dosing or a sustained release formulation especially that Curcumin is rapidly degraded. However, we were trying to monitor if this initial dose would affect the oxidative and inflammatory processes
especially that it was applied locally and immediately post injury thus we assumed a local treatment regimen would be more effective than the oral or the systemic regimen.

Thus, we believe that Curcumin has a great potential in CNS injury due its potential as an anti-inflammatory and anti-oxidant, although longer term investigation are necessary to evaluate the sustainability of Curcumin effects in CNS injury.
CONCLUSION

The results presented here reflect a role of Curcumin in alleviating oxidative damage and inflammation following SCI via lowering MDA, TNF α and IL-6 and elevating TAC. At 7 days following treatment we noticed that Curcumin exerted better effect; however, we believe that further modifications to our studies should be implemented in future investigation. Overall in our current model, local treatment regimen (200 mg / kg) immediately after injury gave better results than Curcumin as a dietary supplement, suggesting that although Curcumin as a dietary supplement may have beneficial effects in general by increasing TAC; however, these benefits are not enough to reverse or reduce the inflammatory process and parenteral administration of Curcumin is needed.
FUTURE RECOMMENDATIONS

Cumulative studies demonstrate a beneficial therapeutic potential of Curcumin following SCI (Cemil et al., 2010; Kavakli et al., 2011; Machova et al., 2015; Yuan et al., 2015). Our study has shown that curcumin might alleviate the consequences of oxidative and inflammatory processes which are two main hallmarks of secondary spinal cord injury.

Many mechanisms have been proposed for its beneficial role yet a verification of the mechanism through which it affects its targets has not been fully described or validated where some recent reports have described Curcuminoids as pan assay interference compounds (PAINS) (Baell and Walters 2014). Thus understanding Curcumin and its mechanism of action towards its targets should be a priority in the ongoing research evaluating its efficacy and potential.

To get results that are more descriptive for the role of Curcumin in SCI we recommend that a new Curcumin delivery system as liposomal curcumin should be used for local treatment regimen. Liposomal Curcumin formulation demonstrated a remarkable enhancement of curcumin bioavailability in cells.

For the dietary regimen we recommend the use of CNB-001 as used by Wu et al. (2011) that would enhance the stability and absorption of curcumin in the biological system. In the present study we measured the total antioxidant capacity; in future investigation we would recommend measuring antioxidant enzymes and investigate which of them is affected by Curcumin treatment following injury as SOD which was found to be elevated by Curcumin (Kavakli et al., 2011; Liu et al., 2013).

Long term behavioral assessment using motor (inclined plane test), sensory (withdrawal reflex test) and sensorimotor tests (Grid walk test) is a future direction regarding our study
to detect if Curcumin dietary supplement or local treatment regimen would manifest sustained motor improvement.

Measuring markers for astrogliosis and axonal sprouting as GFAP and GAP 43 (Growth Associated Protein 43) respectively is an important future investigation for the effect of Curcumin on the environment surrounding the injury site and whether Curcumin will contribute to enhancement of neurological recovery.

Detecting time points before 1 day and between 1 day and 7 days would reflect a clearer idea on Curcumin’s effect in SCI. However, in this study our main scope was comparing between the dietary and local regimens so 7 days and beyond, in our view was suitable for the dietary supplement effect to be investigated.

One of the major obstacles impeding Curcumin’s progress is its low bioavailability, rapid metabolism and poor absorption as well (Kong 2013). For this purpose Curcumin nano-formulations have been developed to enhance its bioavailability and efficacy. This included liposomes, micelles, nanocrystals and others (Rachmawati et al., 2013).

Our results shows that Curcumin might exhibit an antioxidant and anti-inflammatory potential in SCI. Local treatment regimen demonstrated better results than diet supplement; however, we believe that further investigation is needed to unravel the mechanism by which Curcumin acts towards its targets.
REFERENCES


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APPENDIX

1) MDA 1 day:

Results are expressed as mean ± standard deviation

Sig. = Significant , Sign. = Significance, N.S. = Not significant

Table 1: One way ANOVA

<table>
<thead>
<tr>
<th>Marker</th>
<th>1 Day</th>
<th>p-value</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>414.83±126.8</td>
<td>835.67±61.5</td>
<td>493.60±75.2</td>
</tr>
</tbody>
</table>

Statistical test used: One-way ANOVA

$p$-value ≤ 0.05 considered statistically significant (95% confidence interval).

Table 2: Dunnett Post Hoc to identify variables that made significant change

<table>
<thead>
<tr>
<th>Marker</th>
<th>1 Day</th>
<th>p-value</th>
<th>Sig.</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>414.83±126.8</td>
<td>SCI</td>
<td>835.67±61.5</td>
<td>0.003</td>
<td>Significant</td>
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<tr>
<td>SCI+CUR</td>
<td>493.60±75.2</td>
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<td></td>
<td>0.611</td>
<td>N.S</td>
</tr>
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</table>

Statistical test used: Dunnett post Hoc test

$p$-value ≤ 0.05 considered statistically significant different from control (95% confidence interval).

Table 3: Independent t-test

<table>
<thead>
<tr>
<th>Marker</th>
<th>1 Day</th>
<th>p-value</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCI</td>
<td>835.67±61.5</td>
<td>SCI+CUR</td>
<td>493.60±75.2</td>
</tr>
</tbody>
</table>

Statistical test used: Independent t-test

$p$-value ≤ 0.05 considered statistically significant different (95% confidence interval).
2) MDA 7 days

Table 4: One way ANOVA

<table>
<thead>
<tr>
<th>Marker</th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>SCI</td>
<td>SCI+CUR</td>
<td>SCI+DIET</td>
<td>p-value</td>
<td>Sig.</td>
</tr>
<tr>
<td>MDA</td>
<td>414.83±126.78</td>
<td>480.73±121.14</td>
<td>245.38±36.88</td>
<td>406.32±145.71</td>
<td>0.047</td>
<td>Significant</td>
</tr>
</tbody>
</table>

Statistical test used: One-way ANOVA

*p-value ≤ 0.05 considered statistically significant (95% confidence interval).

Table 5: Dunnett Post Hoc to identify variables that made significant change

<table>
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<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>SCI</td>
<td>SCI+CUR</td>
<td>SCI+DIET</td>
<td>p-value</td>
<td>Sig.</td>
</tr>
<tr>
<td>MDA</td>
<td>414.83±126.78</td>
<td>480.73±121.14</td>
<td>245.38±36.88</td>
<td>406.32±145.71</td>
<td>0.014</td>
<td>N.S</td>
</tr>
</tbody>
</table>

Statistical test used: Dunnett post Hoc test

*p-value ≤ 0.05 considered statistically significant different from control (95% confidence interval).

Table 6: Dunnett Post Hoc to identify variables that made significant change

| Marker | 7 Days |        |        |        |        |        |        |        |
|--------|--------|--------|--------|--------|--------|--------|--------|
|        | SCI    | SCI+CUR| SCI+DIET|        |        |        |        |        |
|        | Mean ±SD| p-value| Sig.   | Mean ±SD| p-value| Sig.   | Mean ±SD| p-value| Sig.   |
| MDA    | 480.73±121.14 | ----- | ----- | 245.38±36.88 | 0.014  | Sign.  | 406.32±145.71 | 0.563  | N.S    |
| SCI    | 480.73±121.14 | ----- | ----- | 480.73±121.14 | 0.014  | Sign.  | 480.73±121.14 | 0.563  | N.S    |
| SCI+CUR| 245.38±36.88 | 0.014 | Sign.  | 245.38±36.88 | ----- | ----- | 245.38±36.88 | 0.137  | N.S    |
| SCI+DIET| 406.32±145.71 | 0.563 | N.S    | 406.32±145.71 | 0.137  | N.S    | 406.32±145.71 | ----- | ----- |

Statistical test used: Dunnett post Hoc test

*p-value ≤ 0.05 considered statistically significant different (95% confidence interval).
3) TAC 1 day

Table 7: One way ANOVA

<table>
<thead>
<tr>
<th>Marker</th>
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<td>Control</td>
<td>SCI</td>
<td>SCI+CUR</td>
<td>p-value</td>
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<td>TAC</td>
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<td>0.46±0.21</td>
<td>0.54±0.13</td>
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</table>

Statistical test used: One-way ANOVA

*p-value ≤ 0.05 considered statistically significant (95% confidence interval).

Table 8: Dunnett Post Hoc to identify variables that made significant change

<table>
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<td></td>
<td>Control</td>
<td>SCI</td>
<td>p-value</td>
</tr>
<tr>
<td>TAC</td>
<td>5.66±0.94</td>
<td>0.46±0.21</td>
<td>0.0001</td>
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Statistical test used: Dunnett post Hoc test

*p-value ≤ 0.05 considered statistically significant different from control (95% confidence interval).

Table 9: Independent t-test

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<td>SCI+CUR</td>
<td>p-value</td>
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<tr>
<td>TAC</td>
<td>0.46±0.21</td>
<td>0.54±0.13</td>
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Statistical test used: Independent t-test

*p-value ≤ 0.05 considered statistically significant different (95% confidence interval).
4) **TAC 7 days**

### Table 10: One way ANOVA

<table>
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<tbody>
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<td>Control</td>
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<td>SCI+CUR</td>
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<td>p-value</td>
<td>Sig.</td>
</tr>
<tr>
<td>TAC</td>
<td>5.66±0.94</td>
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<td>0.0001</td>
<td>Significant</td>
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</table>

Statistical test used: **One-way ANOVA**

*p*-value ≤ 0.05 considered statistically significant (95% confidence interval).

### Table 11: Dunnett Post Hoc to identify variables that made significant change

<table>
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<tr>
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<td>p-value</td>
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<td>Sign.</td>
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Statistical test used: **Dunnett post Hoc test**

*p*-value ≤ 0.05 considered statistically significant different from control (95% confidence interval).

### Table 12: Dunnett Post Hoc to identify variables that made significant change

<table>
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<td>Sig.</td>
<td>Mean ±SD</td>
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<td>3.06±1.40</td>
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<tr>
<td>SCI+CUR</td>
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<td>Sign.</td>
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<tr>
<td>SCI+DIET</td>
<td>5.81±0.83</td>
<td>0.0001</td>
<td>Sign.</td>
<td>5.81±0.83</td>
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</table>

Statistical test used: **Dunnett post Hoc test**

*p*-value ≤ 0.05 considered statistically significant different (95% confidence interval).
5) TNF α 7 days

Table 13: One way ANOVA

<table>
<thead>
<tr>
<th>Marker</th>
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<tr>
<td></td>
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<td>SCI+CUR</td>
<td>SCI+DIET</td>
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<td>TNF alpha</td>
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<td>Significant</td>
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Statistical test used: One-way ANOVA

*p-value ≤ 0.05 considered statistically significant (95% confidence interval).

Table 14: Dunnett Post Hoc to identify variables that made significant change

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<tr>
<td>TNF alpha</td>
<td>17.52±1.94</td>
<td>56.09±7.53</td>
<td>18.29±3.76</td>
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Statistical test used: Dunnett post Hoc test

*p-value ≤ 0.05 considered statistically significant different from control (95% confidence interval).

Table 15: Dunnett Post Hoc to identify variables that made significant change

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<td>SCI+DIET</td>
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<td>TNF alpha</td>
<td>56.09±7.53</td>
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<tr>
<td>SCI</td>
<td>56.09±7.53</td>
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<tr>
<td>SCI+CUR</td>
<td>18.29±3.76</td>
<td>0.0001</td>
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<td></td>
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</tr>
<tr>
<td>SCI+DIET</td>
<td>27.17±5.50</td>
<td>0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical test used: Dunnett post Hoc test

*p-value ≤ 0.05 considered statistically significant different (95% confidence interval).
6) IL-6 1 day

**Table 16: One way ANOVA**

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<tr>
<th>Marker</th>
<th>1 Day</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>SCI</td>
<td>SCI+CUR</td>
<td>p-value</td>
<td>Sig.</td>
</tr>
<tr>
<td>IL-6</td>
<td>53.23±5.55</td>
<td>327.99±58.93</td>
<td>244.81±90.51</td>
<td>0.009</td>
<td>Significant</td>
</tr>
</tbody>
</table>

Statistical test used: One-way ANOVA  
*p*-value ≤ 0.05 considered statistically significant (95% confidence interval).

**Table 17: Dunnett Post Hoc to identify variables that made significant change**

<table>
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<tr>
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<th>1 Day</th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>SCI</td>
<td>p-value</td>
<td>Sig.</td>
<td>SCI+CUR</td>
</tr>
<tr>
<td>IL-6</td>
<td>53.23±5.55</td>
<td>327.99±58.93</td>
<td>0.008</td>
<td>Significant</td>
<td>244.81±90.51</td>
</tr>
</tbody>
</table>

Statistical test used: Dunnett post Hoc test  
*p*-value ≤ 0.05 considered statistically significant different from control (95% confidence interval).

**Table 18: Independent t-test**

<table>
<thead>
<tr>
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<th>1 Day</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCI</td>
<td>SCI+CUR</td>
<td>p-value</td>
<td>Sig.</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>327.99±58.93</td>
<td>244.80±90.51</td>
<td>0.315</td>
<td>Not significant</td>
<td></td>
</tr>
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Statistical test used: Independent t-test  
*p*-value ≤ 0.05 considered statistically significant different (95% confidence interval).
7) IL-6 7 days

Table 19: One way ANOVA

<table>
<thead>
<tr>
<th>Marker</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>IL-6</td>
<td>53.23±5.55</td>
</tr>
</tbody>
</table>

Statistical test used: One-way ANOVA

p-value ≤ 0.05 considered statistically significant (95% confidence interval).

Table 20: Dunnett Post Hoc to identify variables that made significant change

<table>
<thead>
<tr>
<th>Marker</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>IL-6</td>
<td>53.23±5.55</td>
</tr>
</tbody>
</table>

Statistical test used: Dunnett post Hoc test

p-value ≤ 0.05 considered statistically significant different from control (95% confidence interval).

Table 21: Independent t-test

<table>
<thead>
<tr>
<th>Marker</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCI</td>
</tr>
<tr>
<td>IL-6</td>
<td>349.56±43.17</td>
</tr>
</tbody>
</table>

Statistical test used: Independent t-test

p-value ≤ 0.05 considered statistically significant different (95% confidence interval).
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- Compression of cord: Burst fractures
- Dislocation: Displacement of vertebra
- Contusion: Displacement and damage of cord
- Ischemia-reperfusion injury: aortic cross-clamp
- Transection of the T4 spinal cord: Partial or complete separation
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