CHAPTER 3

BIRM ATTENUATES PAIN IN ANIMAL MODELS OF CHRONIC CONSTRICTION INJURY-INDUCED PERIPHERAL NEUROPATHY AND IN STREPTOZOTOCIN INDUCED DIABETIC PERIPHERAL NEUROPATHY IN RATS

INTRODUCTION

Neuropathic pain (NP) is a chronic pain condition and is heterogenous in nature. It is defined as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" (Treede *et al.*, 2008). It is considered to arise from damage to nerves due to tumors, diabetic neuropathy, shingles (herpes zoster), complex regional pain syndrome, AIDS, multiple sclerosis, hypoxia or stroke (Mika, 2008). Nociceptive pain which acts as the alert system is easily identified in an organism due to its biological advantage but it becomes difficult in the case of neuropathic pain since it is a result of pathological process representing a disordered regenerative response to neuronal damage (Wallace and Rice, 2008).

Neuropathic pain is highly prevalent with 1.5 - 8.0% of the population getting affected worldwide (Keizer, 2009). It greatly impairs quality of life, and has a high economic impact on society. The institute of medicine reports that at least 116 million American adults suffer from chronic pain and estimates for people suffering from neuropathic pain are as high as 17.9% (von Hehn *et al.*, 2012). Symptoms of neuropathic pain are often severely debilitating such as allodynia, hyperalgesia, spontaneous pain, as well as behavioral disabilities.

It often becomes difficult for patients of NP to exactly describe the pain sensation felt by them since it is different from the nociceptive pain. Clinically neuropathic pain is characterized by the spontaneous pain which may be stimuli independent or dependent (evoked pain) reflecting the hyperexcitability in the nervous system (Bennett, 1994). Patients often complain of burning or stabbing pain which may be either localized or diffused. The mechanism underlying stimulus-independent pain is thought to reflect an increased discharge in sensitized C-nociceptors, occurring due to hyperactivity in sensitized receptors associated

with large myelinated A fibers and giving rise to burning sensation (Jensen *et al.*, 2001). Pain experienced by NP patients may be mix of nocicpetive and neuropathic pain sensation and may have immediate or delayed onset post injury.

Abnormal sensations like allodynia, hyperalgesia, paraesthesia and dysesthesia are the major clinical findings which may overshadow minimal sensory loss occurring in NP (Scadding, 2003).

Sensory loss: Partial or complete loss of afferent sensory function is the essential part of NP. It may involve all sensory modalities, but a loss of spinothalamic functions which includes cold, warmth, pinprick sensation is considered to be crucial (Jensen, 1995). Sensory loss is often difficult to detect with bed-side methods and hence quantitative methods are employed to detect minor changes (Lindblom, 1994; Jensen and Lenz, 1995).

Allodynia: Pain response to non noxious stimuli is termed as allodynia. It refers largely to pain evoked by Aβ-fibers or low-threshold Aδ- and C-fibers (Sandkuhler, 2009).

Hyperalgesia: Increased pain sensitivity to noxious stimuli is termed as hyperalgesia. Hyperalgesia may include both a decrease in threshold and an increase in suprathreshold response (IASP). Primary hyperalgesia occurring at the site of injury is often the result of the sensitization of nociceptive nerve endings. Whereas, secondary hyperalgesia occurring in an area adjacent to or remote of the site of injury is not as a result of sensitization of nociceptive nerve endings but due to changes in processing of sensory information in the central nervous system (CNS) (Sandkuhler, 2009).

Paraesthesia: It is abnormal but non-painful sensation often described as pins and needles and can be spontaneous or evoked. Spontaneous bursts of activity in Aβ fibers may be the reason for this sensation (Jensen *et al.*, 2001).

Dysesthesia: It is an abnormal unpleasant but not necessarily painful sensation. It can be spontaneous or provoked by external stimuli. These sensations are thought to be due to sensitization of C-nociceptros (Jensen *et al.*, 2001).

Abnormal spread of pain has been observed in NP following peripheral and central lesions where patients often experience a circular spreading sensation following single punctuate stimulation. This abnormal radiation is thought to be related to changes in spinal wide dynamic range (WDR) (Dubner, 1991). WDR cells are characterized by small receptive

zones that gets excited by non-noxious stimuli (such as touch, gentle pressure) surrounded by larger zone from which noxious stimuli (such as pinch, firm pressure, temperature > 45 °C) can evoke neuronal discharge. Since these large receptive zones are overlapping, noxious stimulus activates WDR neurons and increases the stimulus intensity resulting in activation of further WDR neurons (Jensen *et al.*, 2001).

There are also instances where patients experience wind-up like pain and after sensations long after the stimulus have been withdrawn reflecting neuronal discharges in WDR neurons (Mendell and Wall, 1965; Lindblom, 1994).

Mechanism of NP

A number of peripheral and central pathophysiological processes after nerve injury have been described through animal studies and they are thought to be the basis of mechanisms underlying neuropathic pain (Romanelli and Esposito, 2004; Bridges, 2001). Generally observed altered sensitivity characteristics in NP is the result of change in function, chemistry and structures of neurons. Peripheral sensitization acts on the peripheral nociceptors whereas central sensitization takes place at various levels right from dorsal horn to the brain. Along with these processes, abnormal interactions between the sympathetic and sensory pathways contribute to mechanisms mediating neuropathic pain (Millan, 1999; Zhang *et al.*, 2004).

Peripheral mechanisms involves ectopic discharges and emphatic conduction, alteration in ion channel expression, collateral sprouting of primary afferent neurons and sympathetic neurons in the dorsal root ganglion (DRG) and nociceptor sensitization. Central mechanisms includes central sensitization, spinal reorganization, cortical reorganization and changes in inhibitory pathways. There are chances where a combination of above or any one factor may contribute in generation of NP and hence it would be inappropriate to generate or unify hypothesis of pathophyisology for all NP conditions (Bridges *et al.*, 2001).

Peripheral Mechanisms

<u>Ectopic discharges and ephaptic conduction:</u> Under normal conditions, rarely does it happen that primary afferent neurons reach the firing threshold in absence of any stimulus. But, it has been observed that following nerve injury, there is increase in the level of spontaneous firing in the afferent neurons (Wall and Gutnick, 1974). This increase in spontaneous firing is termed as ectopic discharge and is observed in patients suffering from NP. During the resting stage, approximately 10% of A-fibers exhibit sub-threshold membrane oscillations but

following nerve injury increase in membrane oscillations of both A- and C- fibers is observed (Amir *et al.*, 1999; Martini, 2000) leading to increase in ectopic firing. It is also observed that ectopic activity play a critical role in development of hyperalgesia, allodynia and ongoing pain associated with nerve injury in animal models as well as in clinical conditions too (Gracely *et al.*, 1992; Amir and Dedor, 2000; Liu *et al.*, 2000). Ectopic firing also alters the expression of sodium and calcium channels under NP conditions leading to increase in the excitability of the neurons and subsequently resulting in spontaneous pain and central sensitization (Devor *et al.*, 1989; Matzner and Devor, 1994; Baccei and Kocsis, 2000)

Collateral sprouting: Sprouting of collateral fibers from sensory axons in the skin into denervated areas was observed in certain cases like nerve crush injuries (Devor *et al.*, 1979) and post chronic constriction injury (CCI) - an animal model of NP (Ro *et al.*, 1996). However, pain behavior was found to be unaffected by collateral sprouting (Kingrey and Vallin, 1989). Nerve growth factor (NGF) from sources within the skin such as keratinocytes, immune cells is found to be responsible for axon sprouting under NP conditions (Diamond *et al.*, 1992; Ro *et al.*, 1996).

Coupling between the sympathetic nervous system and the sensory nervous system: It has been observed that abnormal contact develops between sympathetic nervous system and sensory nervous system following peripheral nerve injury enhancing sensitivity to catecholamines in NP patients (Janig *et al.*, 1996). Sympathetic sprouting observed in various animal models such as spinal nerve ligation (SNL) (Chung *et al.*, 1993) and CCI (Ramer and Bisby, 1997) could be involved in the formation and maintenance of abnormal excitation arising from the DRG (Devor *et al.*, 1994).

Central Mechanisms

Spinal cord - anatomical reorganization: Injury to peripheral nerve causes re-organization of spinal cord and causes sprouting of primary afferent neuron fibers (Aβ- and C- fibers) leading to misinterpretation of information within the spinal cord. Low threshold sensory information gets interpreted as nociceptive giving rise to allodynia post peripheral nerve injury (Bridges *et al.*, 2001).

Spinal cord - hyperexcitability: Persistent inflammation leads to a process called central sensitization where sustained stated of hyperexcitability of dorsal horn neurons is developed (Wall, 1991; Coderre *et al.*, 1993). Central sensitization causes lowering of activation

thresholds of spinal neurons which contribute to development of hyperalgesia through a process called 'windup' where continual low frequency stimulation of C fiber afferents leads to an increase response in the dorsal horn cell (Mendell, 1966; Wall and Woolf, 1986).

Neurotransmitters such as glutamate, Substance P (SP) and brain derived neurotrophic factor (BDNF) get released by central terminals of primary nociceptive afferents in the dorsal horn of the spinal cord in response to pain stimuli. Activation of N-methyl-D-aspartate (NMDA) receptor causes the delivery of repetitive and high-frequency stimulation to primary nociceptive afferents thus amplifying and prolonging the responses of spinal dorsal horn neurons. The contact between neurotransmitters and receptors produce an increase of intracellular Ca^{2+} and cAMP concentrations, which activates protein kinases responsible for signalling cascade that modulates gene transcription i.e. c-fos, c-jun (Willis, 2002; Ji *et al.*, 2003).

Changes in inhibitory pathways: Inefficiency of endogenous inhibitory pathways can also lead to neuropathic pain. The spinal pain transmission system originating from brainstem centers located at the periaqueductal gray and the locus ceruleus is under continuous inhibitory control (Zimmermann, 2001) The γ-aminobutyric acid (GABA) pathway forms a major inhibitory neurotransmitter system in the CNS. Weaker inhibition of this pathway leads to allodynia (Yaksh, 1989) suggesting a role for GABA in modulating the response to peripheral nerve injury. Decreased levels of GABA have been observed in nerve injured rats (Stiller *et al.*, 1996).

Role of Immune system in Neuropathic pain

Immune system plays a critical role in initiating and maintaining neuropathic pain. These non-neuronal cells of immune system release mediators that are recruited at the site of injury or inflammation and might play an important role in initiating and modulating activity in primary afferent nociceptors. Evidence from animal models of NP has proven the role cytokines (IL-1, IL-6), TNF-α, bradykinin, prostanoids and others in pathogenesis of NP (Opree and Kress, 2000; Manning, 2006). Immune mechanisms taking place in peripheral and central nervous system should be looked in separately. Peripheral immune mechanisms include mast cells, neutrophils, macrophages, T cells and cytokines whereas central immune mechanisms include microglia and astrocytes (Moalem and Tracey, 2006).

Peripheral Immune Mechanisms: A variety of immune receptors are present in nociceptors. Any damage/injury to nerve evokes a cascade of immune responses. It leads to macrophage

infiltration, T cell activation and increased expression of proinflammatory cytokines. Schwann cells which get denervated in the process might communicate with intact nociceptors in the same vicinity. Macrophages are recruited through secretion of leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 (MCP-1) (Sugiura *et al.*, 2000). Proinflammatory cytokines initiate the early immune response by communicating between immune cells and nitric oxide (NO) and reactive oxgen species (ROS) and increase the nerve excitability, damage myelin and even alter the blood nerve barrier leading to edema and infiltration of immune cells, antibodies and other immune products (Mizisin *et al.*, 1990; Greenacre *et al.*, 1997; Leem and Bove, 2002). It is evident from animal studies that increased expression of IL-1β leads to increased expression of nerve growth factor (NGF) which goes on to sensitize nociceptors (Kanaan *et al.*, 1998) and TNF-α which is considered as a culprit in NP is also able to initiate activity in nociceptors leading to hyperalgesia (Sorkin *et al.*, 1997).

Central Immune Mechanisms: The final common pathological outcome in NP condition is enhanced excitatory transmission within the dorsal horn leading to pain (Ellis and Bennett, 2013). Dorsal horn, like peripheral nerves contain many immune cells such as glial-derived satellite cells (microglia and astrocytes), dendritic cells, macrophages and endothelial cells in proximity to the cell bodies of sensory neurons (Olsson, 1990). Enhanced excitability occurs via a complex four-way communication between primary afferent terminals, dorsal horn neurons, astrocytes and microglia. Microglia and astrocytes are major contributors for release of multiple inflammatory mediators, neuromodulators and growth factors.

Nerve injury causes cytokine such as TNF-α to enhance the amplitude of glutamate-induced excitatory currents, while IL-1β along with enhancing excitatory synaptic transmission, reduces inhibitory transmission (Kawasaki *et al.*, 2008). Released Cathepsin S cleaves neuronal fractalkine with the resultant soluble fractaline stimulating and further activating microglia in a positive feedback loop (Clark *et al.*, 2009; Clark *et al.*, 2010). Brain derived neurotrophic factor (BDNF) released from microglia cells alters the expression of a key transporter, KCC2, which regulates the anion gradient across neuronal membranes causing an anion gradient shift, reducing hyperpolarizing effects of GABA which further results in disinhibition (Coull *et al.*, 2005). Transmission at the level of dorsal horn is under strong descending control from the rostral ventromedial medulla (RVM) region of brainstem. RVM may have facilitatory and inhibitory components. Wei *et al.* (2008) have demonstrated that

there is a microglial and astrocytic reaction within the RVM, contributing to descending facilitation and enhanced pain related hypersensitivity after nerve injury.

Animal models to study neuropathic pain

Among the types of chronic pain prevailing, neuropathic pain and cancer pain are two of the most difficult types of pain to treat. Although pain can be studied in primates and other mammals, rodents are the far most widely used animals of choice. Whatever progress has been made in recent years in understanding the underlying mechanism of neuropathic pain, it is available from the clinically relevant animal models used to study these painful conditions. Models of neuropathic can be divided into central pain, peripheral injury, peripheral neuropathy induced by disease, cancer pain and cellular models.

Brief information about the models studied under above mentioned categories is mentioned here. However, animal models of our interest of peripheral neuropathy namely chronic constriction injury and diabetes induced neuropathy are discussed in detail in subsequent topics.

Central pain models: They are mainly based on spinal cord injury (SCI).
Weight –drop or contusion model (Allen technique): It is the oldest and most widely used SCI model. SCI is produced by dropping a weight on the surgically exposed spinal dorsal surface at the lower thoracic-lumbar level (Allen, 1911; Balentine, 1978). This injury to spinal cord results in severe paraplegia and development of complete segmental necrosis.

Photochemical SCI model: This method employs use of intravenous administration of erythrosin B, a photosensitizing dye. Upon excitation by an argon ion laser at the exposed vertebrate location, it produces vessel occlusion and parenchymal tissue damage at the endothelial surface of spinal cord vessels (Watson *et al.*, 1986). Autotomy, mechanical and cold allodynia accompanied with hyperalgesia are observed in animals post spinal cord injury (Hao *et al.*, 1991).

Excitotoxic spinal cord injury (ESCI): This method employs intraspinal injection of neurochemicals such as quisqualic acid (QUIS), an AMPA-metabotropic receptor agonist (Yezierski and Park, 1993), glutamate, NMDA acid, kainic acid (Wilcox, 1988; Aanonsen and Wilcox, 1989), serotomin, tryptamine (Larson and Wilcox, 1984). They have been reported to cause long lasting spontaneous pain (detected by excessive grooming), mechanical allodynia and thermal hyperalgesia in rats.

Peripheral nerve injury models: Peripheral neuropathic pain is a complex syndrome resulting from damage to the peripheral nervous system due to trauma, compression, neurotoxins, infection, immune and metabolic diseases, tumors, vitamin, deficiencies and other causes (Wang and Wang, 2003). A number of animal models have been developed to study the conditions that simulate human peripheral neuropathic conditions (Figure 1).
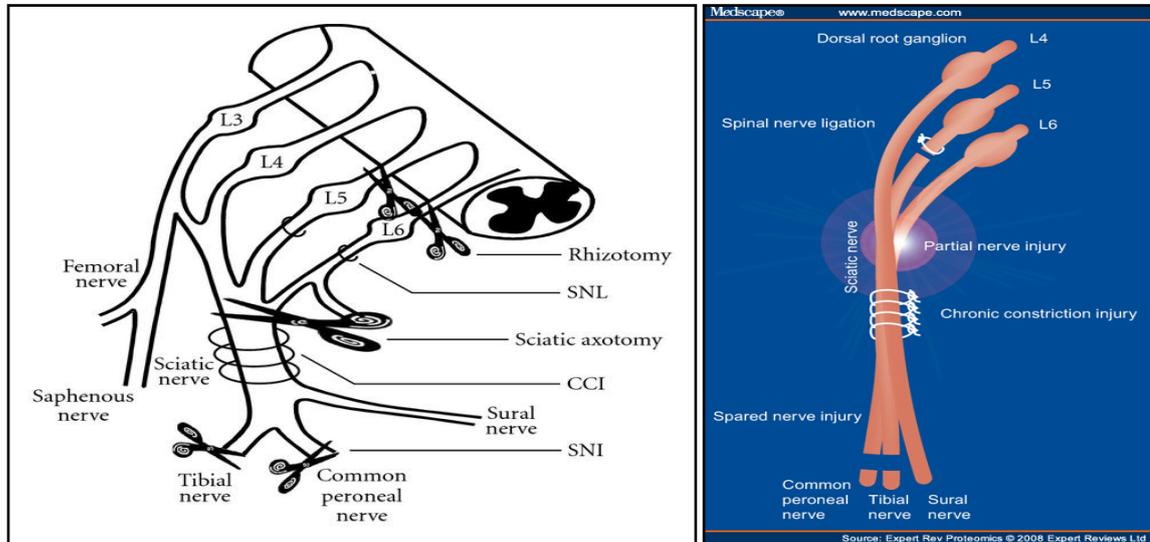

Figure 1: Schematic of the major animal models of nerve injury. SNL: spinal nerve ligation, usually L5 or L5 and L6; CCI: chronic constriction injury consists of loose ligations of the sciatic nerve; SNI: spared nerve injury, consists of section of the tibial and the peroneal branches leaving the sural intact. Courtesy http://img.medscape.com/ fullsize/ migrated/ 586/220/epr586220.fig1.gif

Neuroma Model: Complete transaction and ligation at multiple locations such as siatic nerve leads to development of neuroma consisting of regenerative nerve sprouting in all directions responsible for autotomy (Devor and Wall, 1976; Wall *et al.*, 1979; Amir and Devor, 1993).

Chronic constriction injury model (CCI or Bennett model): In this model, sciatic nerve is loosely tied with four chromic gut ligatures at the mid thigh level (Bennett and Xie, 1988). CCI rats shows behavioral signs of spontaneous pain (includes mild to moderate autotomy, excessive licking, safe guarding and limping of ipsilateral paw), thermal hyepralgesia and mechanical allodynia, lasting for over a duration of two months (Bennett and Xie, 1988; Attal *et al.*, 1990).

Parital sciatic nerve ligation model (PSL or Seltzer model): The model involves ligation of the ipsilateral sciatic nerve at the high-thigh level, so that 1/3-1/2 thickness of the sciatic nerve is trapped in the ligature. This model simulates causalgia resulting from partial nerve

injury in humans and shows signs of mechanical allodynia and hyperalgesia to thermal and mechano-noxious stimuli accompanied with spontaneous pain behavior in the form of paw guarding and licking in bilateral pattern (Seltzer *et al.*, 1990).

L5/L6 spinal nerve ligation model (SNL): This model reported by Kim and Chung (1992) simulates causalgia in humans. L5 and L6 spinal nerves are unilaterally and tightly ligated at a location distal to DRG. Behaviour signs like allodynia and hyperalgesia are observed atleast for four months after nerve injury.

L5 spinal nerve ligation: In this model, ligation of L5 spinal nerve leads to lasting hyperalgesia and mechanical allodynia and may provide a useful option for studies involving mice (e.g. gene knock out or transgenic mice) (Kim and Chung, 1992; Fairbanks *et al.*, 2000)

Spared nerve injury (SNI): The SNI model is based on section and ligation of two of the three peripheral branches of the sciatic nerve: the tibial and common peroneal nerves are ligated and the sural nerve remains intact. This co-mingling of distal intact axons with degenerating axons is restricted in this model which makes behavior testing of the non-injured skin territories adjacent to the denervated areas possible. SNI leads to quick sensory hypersensitivity and mechanical as well as thermal hyperlgesia (Decosterd and Woolf, 2000).

Sciatic cryoneurolysis model (SCN): SCN involves freezing of sciatic nerve instead of complete transaction or ligation to produced nerve injury (DeLeo *et al.*, 1994). Pain behaviors such as spontaneous pain behavior, tocuh evoked allodynia lasting for 15-20days are observed within the first 7 days after nerve injury (DeLeo *et al.*, 1994). Advantage using this method is that nerve injury occurring is reversible thus allowing studies of transient nerve injury and the healing process (Willenbring *et al.*, 1995a,b).

Inferior caudal trunk resection model (ICTR): The model involves unilaterally resecting the inferior caudal truck between S3 and S4 nerves (Na *et al.*, 1994; Back *et al.*, 2002). Unlike other models, this model allows behavioral testing in tail instead of hindpaws. Pain behavior such allodynia and hyperalgesia are observed within a day after injury and lasts for several weeks.

Sciatic inflammatory neuritis model (SIN): Considering the fact that inflammation or infection is the major reason behind neuropathies observed in humans, this model involves peri-sciatic immune activation by placing a proinflammatory gut suture (Maves *et al.*, 1993),

dead bacteria or carageenan (Eliav *et al.*, 1999) on the sciatic nerve or injection of zymosan around the siatic nerve (Chacur *et al.*, 2001) to produce neuritis with pain behaviors such as both allodynia and hyperalgesia.

<u>Peripheral neuropathy induced by diseases</u>: Neuropathic pain as a sequellae of shingles and diabetes is common in humans and is seen to persist for many years.

Postherpetic neuralgia model (PHN): Shingles caused due to reactivation of a primary infection with varicella-zoster virus in humans is often followed by postherpectic neuralgia – a condition characterized by the presence of spontaneous and evoked pain (such as burning, aching) (Rowbotham and Fields, 1996). Sadzot-Delvaux *et al.* (1990, 1995) used rat model of latent varicella-zoster virus infection to study persisted allodynia and hyperalgesia observed in postherpetic neuralgia.

Diabetic neuropathic pain model (DNP): Although several models of diabetes such as insulin deficient BB rats (Sima, 1980), NOD mice (Mosseri *et al.*, 2000) insulin resistant ob/ob and db/db mice (Meyerovitch *et al.*, 1991), the Mongolian gerbil (Vincent *et al.*, 1979) and chemically induced models (Lee *et al.*, 1990; Courteix *et al.*, 1994) are available, the chemically induced model of diabetes (using alloxan or streptozotocin) is preferred to study peripheral neuropathy. The widely used method employs single i.p. injection of streotozotocin in rats. Streptozotocin is known to kill insulin-secreting islet cells. This treatment results in hyperglycemia followed by long lasting thermal and mechanical hyperalgesia and cold and thermal allodyia (Forman *et al.*, 1986; Courteix *et al.*, 1993; Courteix *et al.*, 1994).

<u>Cancer pain models:</u> Cancer pain – a common symptom in cancer patients is increasingly becoming a bigger problem affecting the quality of life. Cancer related pain may be caused by tumor infiltration or compression of nerve, plexus, or roots, immunoreactive and pronociceptive substances released from tumors or by treatment (chemotherapy, radiation or surgery) (Vecht, 2000). Attempts are being made to develop animal models of cancer pain to understand the mechanism and provide better treatments.

Chemotherapy-induced peripheral neuropathy models: This model involves administration of chemotherapeutic agents in animals. These agents produce neuropathy which can be used to study causes, prevention and treatment of their neurotoxicity. Vincristine-induced peripheral neuropathy model (VIPN) (Aley *et al.*, 1996), taxol-induced peripheral neuropathy model (TIPN) (Cavaletti *et al.*, 1995) and Cisplatin-induced peripheral neuropathy model (CIPN)

(Authier *et al.*, 2000) are the three models used for studying neuropathy observed due to chemotherapeutic agents. Mechanical allodynia and hyperalgesia are the common pain behaviors observed in these models.

Cancer invasion pain model (CIP): This model involves implantation of Meth A sarcoma cells around the sciatic nerve in Balb/c mice. It leads to development of allodynia and thermal hyperalgesia as the nerve compression increases with growth in tumor (Shimoyama *et al.*, 2002).

Bone cancer pain models: It is one of the most common cancer-related pain (Mercadante, 1997). Mouse femur bone cancer pain model (FBC)(Schwei *et al.*, 1999), Mouse calcenous bone cancer pain (CBC) (Wacnik *et al.*, 2001) and Rat tibia bone cancer model (TBC) (Walker *et al.*, 2002) are the three models which has been developed to study bone cancer pain and get insight of its mechanism to enable the development of appropriate therapeutic options.

Cellular models: Cell lines are found to be useful for studying the molecular and cellular mechanisms of both acute and chronic pain and may mimic certain aspects of in vivo signal transduction between cells or within a single cell (Wang and Wang, 2003). Primary culture of sensory neurons (Baccaglini and Hogan, 1983; Vasko *et al.*, 1994) and permanent sensory neuron cultures (Platika *et al.*, 1985; Wood *et al.*, 1990; Raymon *et al.*, 1999) are widely used to study the pain mechanisms in *in vitro* conditions.

Studies carried out using these animal models in last decade provides evidence of interactions between neurons, inflammatory immune and immune like glia cells, inflammatory cytokines and chemokines. Peripheral nerve injury provokes a reaction from the immune system and has been observed at various anatomical locations including the injured nerve, the dorsal root ganglia (DRG), the spinal cord and supraspinal sites associated with pain pathways (Austin and Moalen-Taylor, 2010). Emerging lines of evidence have revealed that changes also occur in spinal microglia, the immune cells of the central nervous system (Inoue and Tsuda, 2009). Activation of microglia is a major feature of neuropathic pain and growing evidence suggests that microglia have a causal role in pathogenesis of persistent neuropathic pain and hence a detailed study of the microglial cells and its exact role in central pain seems necessary.

Current treatment options and the need to look for alternate options

Treatment options available for the management of neuropathic pain are as diverse as its etiologies. Hence, neuropathic pain remains poorly managed pathological condition by currently available therapeutics. Non-opioids analgesics (NSAIDs, acetaminophen), opioid analgesics (mu opioids agonists, agonists-antagonists opioids), adjuvant analgesics such as anti-epileptic drugs, tricyclic antidepressants and local anesthetics (lidocaine, bupivacaine) are some of the commonly used options for the treatment of chronic pain. Studies have revealed that current treatments available for neuropathic pain indicate general insensitivity to non-steroidal anti-inflammatory drugs and relative resistance to opioids or potential misuse and risk of addiction to opioids. These treatments have untoward side effects when given at higher doses to obtain adequate analgesia (Dray, 2008).

Researchers around the globe are looking for alternate treatments which can offer adequate analgesia devoid of severe side effects. Plants still remain to be major possible source of new drugs and chemicals. Herbal medicines are most sought after treatment option in the category of alternative treatments. They continue to be the source of lead structures for synthetic modifications and optimization of bioactivity. Due to severe side effects associated with available therapeutic options as mentioned above, medicinal products derived from plants are preferred and are becoming part of the integrative health care systems in industrialized nations (Qadrie *et al.*, 2009). A dramatic increase is seen in the number of patients opting for complementary and alternative medicine and consuming plant extracts from folklore medicine (Smith and Mills, 2001). They are found to be relatively safe.

BIRM: A prospective drug against neuropathic pain

Biological Immune Response Modulator (BIRM) is an oral solution formulated from extracts of Amazonian plant *Solanum dulcamara* by a physician E.Cevallos-Arellano, native of Ecuador. This formulation is considered as a natural remedy for number of ailments (such as AIDS, Cancer) and is consumed as a dietary supplement by Ecuadorian native population (Cevallos, 1994).

Exact mechanisms of the herbal medicine remain elusive. But, most of the herbal medicinal products exert their efficacy/potency through several pathways which include inhibition of cyclooxygenase (COX) and/or lipoxygenase, inhibition of cytokine release, inhibition of elastase or hyaluronidase and may induce antioxidative activity (Cameron *et al.*, 2009a, b). In

line with the above hypothesis, herbal medicinal product of our choice, Biological Immune Response Modulator is thought to exert its potential efficacy through inhibition of COX in therapeutic area of pain and inflammation. Moreover, Jaggi *et al.* (2004) have studied mother tincture of *Solanum dulcamara* the source of BIRM and found that it inhibits production COX-1 and COX-2 but do not inhibit production of leukotriene LTB4 by 5-LOX. Now, as we know, COX plays an important role in pain pathways. However, BIRM remains to be explored for its efficacies in other therapeutic areas except for cancer wherein it has been shown to be efficacious in inhibiting progression of tumor (Dandekar *et al.*, 2003).

Under the circumstances, where current therapeutic options for neuropathic pain are not effective enough, BIRM showing its COX-inhibition properties and COX being mediator of pain, we found it good enough to explore the efficacy of BIRM and study its analgesic effects in animal models of peripheral neuropathic pain.

OBJECTIVE

The objective of this study was to assess analgesic effects of BIRM through its effect on peripheral and central immune system in animal models of peripheral neuropathic pain namely 1. chronic constriction injury model and 2. streptozotocin induced diabetic neuropathy model.

The results from both these models are described hereunder in two separate headings for better comprehension.

1. CHRONIC CONSTRICTION INJURY MODEL OF PERIPHERAL NEUROPATHIC PAIN

Bennett and Xie (1988) reported a model that produces allodynia and hyperalgesia − salient features of peripheral neuropathic pain. It simulates the clinical condition of chronic nerve compression such as that occurring in nerve entrapment neuropathy or spinal root irritation by lumbar disk herniation (Kumar *et al.*, 2014). The nerve injury is created by tying loosely constrictive ligatures around the rat's sciatic nerve. The ligatures evoke intraneural edema as the swelling is opposed against the ligatures leading to strangulation of nerves.

It is well understood that constriction axotomizes nearly all of the nerve's large myelinated axons and a very large majority of its small myelinated axons too. Nerve's unmyelinated axons remain intact. Although the nerve distal to the constriction is full of degeneration, the nerve proximal to the constriction appears normal and there is no evidence of primary

afferent neurons dying (Basbaum *et al.*, 1991). Pain behavior such as allodynia and hyperalgesia develops in 10-14 days post nerve injury and lasts for 2 months. Spontaneous pain and evoked pain sensation observed in CCI rats are similar to those observed in patients with painful peripheral neuropathies. Spontaneous pain is usually observed in animal's home cage. It suddenly lifts the nerve damaged side paw and licks and shakes it. This behavior is similar to that observed in patients experiencing sudden pain (Bennett and Xie, 1988). Spontaneous behavior becomes visible when the axotomized $A\beta$ and $A\delta$ primary afferent neurons quickly begin to discharge spontaneously at high frequencies. The C-fiber neurons those are silent in initial phase of CCI, starts firing after a week.

As discussed earlier, hyperalgesia to heat and mechanical stimuli is observed in CCI. With respect to thermal hyperalgesia, it is evident that a substantial decrease in the temperature is necessary to evoke a nocifensive withdrawal reflex of large duration and is accompanied with licking of paw. Whereas in mechanical hyperalgesia, rats exhibit exaggerated response when hind paw is touched with the point of a pin and the response of the nerve injured side is greatly increased in amplitude and duration (Tal and Bennett, 1993).

Allodynia of two types are generally observed in CCI namely allodynia to mechanical stimuli and to cold stimuli and it is a troublesome symptom in patients. Cold allodynia is very much evident when rat, after CCI surgery repeatedly withdraws the nerve injured side paw and guards it from contact with a cold floor (Bennett and Xie, 1988). Mechanical allodynia is observed when the plantar surface of the hindpaw exposed to light touch of non painful von Frey hairs, is withdrawn with jerk and exaggerated amplitude and duration, accompanied with paw licking. This resembles the pain observed in neuropathic pain patients when abnormal evoked-pain sensation in an area is felt, that does not correspond to the territory of a nerve or dorsal root.

MATERIAL AND METHODS

Animals and housing conditions

Male Wistar rats (180-210g) were procured from CPCSEA and AAALAC approved, Vivarium Facility at GVK Biosciences Pvt. Ltd., Hyderabad, India. They were allowed to acclimatize for a minimum duration of one week prior to surgical intervention. They were housed in groups of three in polypropylene cages under ambient conditions prior to surgery and housed individually post-surgery. Room temperature and humidity were maintained at 22-25 °C and 65-70%, respectively. 12h light/dark cycle was maintained. Standard laboratory

rodent diet and potable drinking water were provided *ad libitum*. To prevent wound infection after a surgical procedure, the surgical area was dusted with streptomycin before suturing the incision in all the animals. The protocols were approved by IAEC as per CPCSEA guidelines. All animal procedures were performed within the ambit of ethical guidelines of CPCSEA.

Chronic constriction injury

The method described by Bennett and Xie (1988) was generally followed. Rats were anesthetized with gaseous anesthetic Isoflurane (Baxter, Germany). The right common sciatic nerve was exposed at the level of the mid-thigh by blunt dissection through the biceps femoris. Proximal to the sciatic's trifurcation, about 12 mm of nerve was freed of adhering tissue and four ligatures (chromic catgut, Johnson and Johnson) were tied loosely around it with an interval of about 1mm among ligatures. The length of nerve thus affected was 6-8 mm long. Great care was taken to tie the ligatures such that the diameter of the nerve was just barely constricted. The desired degree of constriction retarded, but did not arrest, circulation through the superficial epineural vasculature. The incision was closed in layers. To avoid variation, all procedures were performed by the same individual. The wounds healed gradually with no infection after the surgery.

Test compound and treatment regimen

BIRM was a gift from BIRM Inc. (Quito, Ecuador). It is an aqueous extract of dried roots of a plant of the species *dulcamara* (family Solanaceae) grown in Ecuador, and marketed as a greenish-brown suspension with a mild bittersweet smell.

In the present study, BIRM samples from same lot number were used and it was clarified by centrifugation at 10,000g prior to usage as described by Dandekar *et al.* (2003). Gabapentin was obtained from Sigma Aldrich, USA.

Though the recommended minimum dose of BIRM for human consumption, as per the container label, is 4 ml/day, we used BIRM at 4 ml/kg dose based on dose range finding study in *in vivo* experiments (acute inflammatory models). BIRM when administered orally at 4 ml/kg dose level was found to be well tolerated and no observable systemic toxicity in rodents was observed.

Surgically operated CCI rats were randomly selected after assessment of mechanical allodynia and divided into four groups (N=8/group). Drug testing was initiated on day 14 post surgery. Animals from Normal Control (NC) and CCI operated Control (CCI-VC) were

orally administered distilled water throughout the study duration. Animals from BIRM-CCI operated group (CCI- BIRM) were administered BIRM daily at 4 ml/kg dose volume (day 14 to day 28 post surgery) through oral route. Gabapentin (30 mg/kg, p.o.) was administered once on day 14, 21 and 28 post surgery.

Neuropathic pain measurements

Mechanical Allodynia: Mechanical allodynia was assessed by modified Dixon's Up and Down method using a set of von-Frey filaments (0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0 and 15.0 g). It was assessed at 3 hr post first dose on day 14, 21 and day 28 post-surgery.

Briefly, the rats were placed in clear plexiglass chambers on a wire mesh floor and allowed to acclimatize for at least 20 min. The testing was initiated with the 2.0 g filament. The filament was applied once to the mid-plantar surface of ipsilateral and contralateral paw in a perpendicular fashion and until slight buckling occurred for 6-8s. Positive responses included an abrupt withdrawal of the paw from the stimulus or flinching behavior immediately following removal of the stimulus. Stimuli were always presented in a consecutive fashion, whether ascending or descending. In the absence of a paw withdrawal response to the initially selected filament, a stronger stimulus was applied; in the event of paw withdrawal, the previous weaker stimulus was chosen. The test was completed when four measurements were made after the initial change in behavior or after five consecutive negative or four positive responses has occurred. The resulting pattern of positive and negative responses was tabulated using the convention, X= withdrawal; O = no withdrawal and the 50% response threshold was interpolated using the method followed by Chaplan *et al.* (1994)

Data were reported and plotted as 50 % g threshold values and the percentage effect was calculated using 50 % g threshold values using the formula:

$$\% \text{ Protection} = \frac{(50\% \text{ PWT posttreatment } - 50\% \text{ PWT pretreatment })}{(\text{Maximum possible } 50\% \text{ PWT i.e.} 15 - 50\% \text{ PWT pretreatment })}$$

Thermal Hyperalgesia: Thermal response was determined by measuring hind paw withdrawal latency of affected paw employing Hargreaves' plantar test. Rats were allowed to acclimatize within plexiglass enclosures on a clear glass plate maintained at 30°C for 15-30 minutes before testing. A radiant heat source controlled with a timer was focused onto the plantar surface of hind paw encompassing the glabrous skin. On response of paw withdrawal, both heat source and timer were stopped and paw withdrawal latency was recorded. Five

trials, 1-2 minutes apart were conducted and average of three trials excluding maximum and minimum response was taken as paw withdrawal latency. The cut-off limit for exposure was 20 seconds (Hargreaves *et al.*, 1988).

Sample Preparation

On day 28 post surgery (14 days post treatment), the rats (n=4 per group) from NC, CCI-VC and CCI-BIRM were deeply anesthetized with isoflurane and immediately perfused intracardially with 400-500 ml of cold phosphate buffered saline (0.01 M, pH -7.4) followed by 2% paraformaldehyde in 0.01 M phosphate buffer (pH-7.4) through the ascending aorta. Then their lumbar spinal cords (L4-L6 region) were quickly removed. From another set of four animals from each group, the lumbar spinal cord tissues for RT-PCR and western blot analysis were collected and immediately stored at -80°C until analysis.

Western blot - expression of Iba-1 protein the microglia cell marker

Western blot analysis was performed to measure expression of Iba-1 in the spinal cord dorsal horn samples. The protein extracts were separated on 15% polyacrylamide gels by electrophoresis and transferred to nitrocellulose membrane. After the membrane was incubated in blocking buffer (5% nonfat milk in -Tris-buffered saline with Tween 80 -TBST) for 1h at room temperature, they were incubated overnight at 4°C in the presence of anti-Iba-1 antibody (ab5076, Abcam) (1:1000 in 5% nonfat milk in TBST). Protein weights were measured against Precision Plus protein standards (Bio-Rad, Hercules, CA, USA). After being washed in phosphate buffered saline with Tween 20, the membrane was incubated with a secondary antibody –Rabbit anti-goat IgG coupled to horseradish peroxidase (1:3000) (cat # 611620, Invitrogen) for 2h at room temperature. Protein bands were visualized by chemiluminiscence with ECL detection reagent (GE Healthcare-Amersham, Pittsburgh, PA, USA). The membranes were re-probed with antibody to β-actin for use as an internal loading control. Densitometric analysis was carried out using Quantity One software (Bio-Rad)/Alpha Ease FC software, version 4.0.034 (Alpha Innotech), San Leandro CA, and the results were normalized to loading control.

RNA isolation and cDNA synthesis

Total RNA from the lumbar spinal cord was extracted using the standard phenol/chloroform extraction with TRIzol Reagent (Invitrogen) according to the manufacturer's guidelines. Samples were treated with DNase to remove any contaminating DNA (Ambion, USA). Total RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit

(Thermo Scientific, Cat # K1621)). First-strand cDNA was synthesized using total RNA, random hexamer primer (0.2µg/µl) and 10 mM dNTP mix and incubated at 65°C for 5min. After 2min incubation on ice, a cDNA synthesis buffer (5X Reaction buffer) and dithiothreitol (10 mM) was added and incubated at 25°C for 2min. Reverse transcriptase (RevertAid M-MuLV Reverse Transcriptase (200 u/µl)) was added to a total volume of 20 µl and incubated for 10 min at 25°C, 50 min at 42°C, and deactivating the enzyme at 70°C for 15min. All cDNA samples were stored at -80°C until real-time PCR (qPCR) was performed.

<u>Real time PCR</u>

Amplification of the cDNA was performed, in a blinded procedure, using SYBR® Select Master Mix kit (Life Technologies/ABI) in MicroAmp® 96 well plate (Life Technologies/ABI) on a ABI Step-one Real Time PCR machine (ABI). The reaction mixture (50 µl) was composed of 2X PCR SYBR green ready mix (containing fluorescent dye SYBR Green, 1.25 mM MgCl2, dNTP and Fas Taq), 2 µl of each forward and reverse primer (100 ng/µl), nuclease free water and 1 µl of cDNA from each sample. Each sample was measured in triplicate. The reactions were initiated with a hot start at 94ºC for 5 min, followed by 40 cycles of 5 s at 94ºC (denaturation), 10 s at 55ºC (annealing), and 10 s at 72ºC (extension). Melt curve analyses were conducted to assess uniformity of product formation, primer-dimer formation and amplification of non specific products. The PCR product was monitored in real time, using the SYBR Green 1 fluoroscence, using the ABI Step-one Real Time PCR system (ABI). Threshold for detection of PCR product was set in the log-linear phase of amplification. The comparative cycle threshold (Ct) method was used for relative quantification of gene expression. The amount of mRNA, normalized to the endogenous control (GAPDH) and relative to a calibrator, was given by $2^{-\Delta(\Delta Ct)}$, with Ct indicating the cycle number at which the fluorescence signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR. The level of the target mRNA was quantified relative to the house keeping gene (GAPDH) using the comparative CT (Δ CT) method. The expression of GAPDH was not significantly different between treatments.

The fold change in the target gene relative to the GAPDH endogenous control gene was determined by the following formula.

$$\text{Fold Change} = 2^{-\Delta(\Delta Ct)}$$

Where:
ΔCt = Ct (target) - Ct (GAPDH)
$\Delta(\Delta Ct)$ = ΔCt (test sample) - ΔCt (calibrator)

List of primer sequence used for q-PCR for gene expression studies (Source: NCBI Database)

Primer	Sequence	Basepair (bp)
TNF-α	F: GATGGGCTGTACCTTATCTACTCCCAGG	151
	R: CCTTAGGGCAAGGGCTCTTGATGGC	
IL-10	F: TAAGGGTTACTTGGGTTGCCAAGCC	149
	R: GCAGCTGTATCCAGAGGGTCTTCAGC	
COX-2	F: CAGTATCAGAACCGCATTGCCTCTG	149
	R: GTGAGCAAGTCCGTGTTCAAGGAGG	
GAPDH	F: CAAGGTCATCCATGACAACTTTGGC	184
	R: CTGGGATGACCTTGCCCACAGCC	

Immunohistochemistry

Spinal cord tissues collected after transcardial perfusion were washed with phosphate buffer and cryopreserved in sucrose gradients i.e., 10, 20 and 30% (prepared in 0.01M phosphate buffer) at 4°C, till the tissues settled at bottom. Frozen tissue was embedded in OCT (Tissue-Tek, Sakura Finetek, USA) and sections were taken at 12 μm thickness with cryotome (Reichert-Jung, Cryocut E Cryostat).

Tissues were immunolabeled using standard immuno-histochemistry methods for microglial localization and Iba1 expression. Briefly cryocut sections were brought to room temperature and air-dried at 37 °C in a hot air oven. Air dried sections were washed with 0.01 M PBS (pH 7.2 - 7.6) buffer thrice for 5 minutes each to remove cryomount present on the sections. Next to the buffer washes, sections were treated with 1%Triton X-100 for membrane permeabilization. Excessive Triton X-100 was removed with three changes of 0.01M PBS buffer. Following membrane permeabilization endogenous peroxidase blocking was performed with the treatment of 1% H_2O_2 for 20 min. Excess of H_2O_2 was removed by three changes of buffer of 5 min each. Non specific proteins were blocked by using 1% normal goat (NGS) serum or in which secondary antibody has raised. Sections in NGS were incubated for 60 min in a moist chamber at room temperature to avoid sections drying. Sections were then incubated in primary antibody (goat polyclonal anti-Iba1 antibody, Abcam) diluted 1:300 in 1% Bovine Serum Albumin (BSA) in buffer at 4 °C for overnight in a moist chamber. Next day sections were kept at room temperature for 20 min before starting further processing. To remove unbound primary antibody, sections were washed with buffer

3 times 5 min each. This was followed by incubation with biotinylated rabbit anti-goat IgG conjugated secondary antibody (Invitrogen, India) at a dilution of 1:200, diluted in 1% BSA in buffer for 60 min at room temperature in moist chamber. Sections were again washed with buffer for three times of 5 min each to remove unbound secondary antibody. Next sections were incubated with Streptavidin HRP Complex (1:200), diluted in 1% BSA in buffer for 1 h at room temperature in moist chamber. Wash in buffer (3 x 5 min each). Sections were visualized in 3'3-Diaminobenzidine tetrahydrochloride - DAB (Sigma Aldrich, USA). Sections were lastly washed with running tap water for 5 min followed by single rinse in distilled water and air dried in hot air oven at 37°C for 45 min. Such completely air dried sections were dehydrate in absolute alcohol, 2 changes of 10 min each followed by clearing in xylene for 10 min and mounted in DPX. From each animal's spinal cord, four to five sections within the L4-L5 region were included in the analysis. At every immunolabeling point, respective control was used.

The sections were observed under Leica DM2500 microscope and the images were captured using EC3 camera utilizing Leica LAS EZ (V 1.6.0) software. The images were quantified using Doc ItLS software (Genei, Bangalore, India) by an observer unaware of experimental conditions. The pixel measurement was used for counting the density-slicing area in the image of the positive area of the dorsal horn of the spinal cord. Then, the fold change in the staining density between NC, CCI-VC and CCI-BIRM was calculated. The criteria for resting and activated microglia were as described previously (Hains and Waxman, 2006). Briefly, the resting morphology is characterized by small compact somata bearing long, thin, ramified processes, and activated morphology is characterized by a process length less than the diameter of the soma compartment. Immunohistochemical data were expressed as the fold change compared with the normal control animals, which were considered to be 100%. All samples from all groups were numbered randomly and blinded observation was carried out to prevent bias.

Statistical Analysis

One way ANOVA test followed by Tukey's multiple comparison test was applied for 50% PWT analysis and 50% PWL analysis. Unpaired Student's t-test was used for analysis of data generated from gene expression studies. $p \leq 0.05$ was considered statistically significant. For ease of reading, the basic statistical values are shown in the text while the more extensive statistical information can be found in the figure legends.

RESULTS

<u>The effect of BIRM on CCI - induced changes in behavioural and neuropathic pain measurements</u>

After CCI surgery, the rats gradually showed the typical signs of allodynia and hyperalgesia such as toe closing, foot eversion and paw licking. There was no significant difference observed in ipsilateral 50% paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) among the groups (p > 0.05) after characterization of CCI.

Mechanical Allodynia

With respect to mechanical allodynia, CCI-vehicle control group showed significant decrease in 50%PWT as compared to normal control group throughout the study duration post CCI induction on days 13, 14, 21 and 28 (P ≤ 0.001). Repeated oral treatment with BIRM (4 ml/kg) as a single dose daily for 14 days showed an average protection of 35% and 38% at 3 h post BIRM administration on day 14 and day 21, respectively. The increase in 50% PWT was significantly higher than CCI-vehicle control group (CCI-VC) on day 14 (p ≤ 0.05) but not on day 21 as analysed by One Way ANOVA followed by Tukey's multiple comparison test. BIRM showed average protection of 70% at 3h post BIRM administration on day 28. This increase in 50% PWT was significantly higher than CCI - vehicle control group (p ≤ 0.001) as analysed by One Way ANOVA followed by Tukey's multiple comparison test (Table 1; Figure 2).

Treatment with Gabapentin at 30mg/kg showed an average protection of 82%-83% at all assessment time. The increase in 50% PWT was significantly higher (P ≤ 0.001) than CCI - vehicle control group as analyzed by One Way ANOVA followed by Tukey's multiple comparison test (Table 1; Figure 2).

Thermal hyperalgesia

With respect to thermal hyperalgesia, CCI-Vehicle control group (CCI-VC) showed significant decrease in 50% PWL throughout the study duration post CCI induction i.e., on days 14, 21 and 28 (p ≤ 0.001). But treatment with BIRM (4 ml/kg/day) showed a significant increase in PWLs at 3h post administration on day 14 (p ≤ 0.05), day 21 (p ≤ 0.001) and 28 (p ≤ 0.001). This increase in PWLs was significantly higher than CCI - vehicle control group as analysed by One-way ANOVA followed by Tukey's multiple comparison test (Table 2; Figure 3).

Treatment with Gabapentin showed an average increase in PWLs at 3 hr post administration day 14, 21 and 28. The increase in PWLs was significantly higher than CCI - vehicle control group as analysed by One-way ANOVA followed by Tukey's multiple comparison test (Table 2; Figure 3).

The effect of BIRM on CCI - induced Iba-1 protein expression

Quantitative analysis of Western blots showed that Iba-1 protein level had significantly increased in lumbar spinal cord tissue on day 28 post CCI surgery ($p \leq 0.001$). Significant reduction in Iba-1 protein level was observed in the CCI group treated with BIRM ($p \leq 0.01$) (Figure 4). These findings in western blot experiment suggest neuroprotective effect of repeated oral treatment of BIRM (4 ml/kg/day) on microglia activation in lumbar spinal cord tissue

The effect of BIRM on CCI – induced COX-2, TNF-α, IL-10 expression in spinal cord

Mean fold increase was observed in mRNA levels of TNF-α (0.022 times) and COX-2 (0.141 times) in lumbar tissue (L4-L5) in CCI-VC animals as compared to normal control (NC) animals. Mean fold change of 0.008 times was observed in mRNA levels of IL10 in animals of CCI-VC group. But treatment with BIRM was able to bring significant reduction in mean fold change in mRNA levels of TNF-α (0.007 times)($p \leq 0.01$) and COX-2 (0.032 times) ($p \leq 0.001$)in lumbar tissue (L4-L5) in CCI-VC animals as compared to CCI-VC group. Similarly, significant increase in fold change in mRNA levels of IL-10 (0.029)($p \leq 0.01$) in CCI-BIRM group was observed as compared to CCI-VC group. (Table: 3; Figure 5) Results were analysed using Unpaired Student's t test.

The effect of BIRM on CCI-induced changes in spinal microglia cells

Immunohistochemistry was performed using the Iba-1 antibody which is known to selectively label activated microglia in nervous tissue. In the normal control sections, a few Iba-1 positive cells could be seen (Figure 6.A) as compared to higher number of Iba-1 positive cells in CCI-VC and CCI-BIRM sections (Figure 6.B and 6.C). The morphology of microglial cells in normal control sections exhibited the resting stage to some extent, which has small compact somata bearing long, thin, ramified processes (Figure 6.A, arrow marked). Although the thin, ramified processes are not clearly visible in our sections, soma diameter is found to be much smaller and compact as compared to activated microglial cells in CCI-VC and CCI-BIRM groups. Microglia exhibited an activated phenotype, showing hypertrophy and retraction of cytoplasmic processes in the sections of CCI rats. Compared to normal

control rats, a significant shift from resting to activated morphology was found in CCI rats. Chronic treatment of BIRM (4 ml/kg) for 14 days was found to reduce the proportion of the activated phenotype in microglial cells (Figure 6.C). Quantification of Iba-1 immunoreactivity in lumbar dorsal horn shows significant increase in Iba-1 immunoreactivity in CCI-VC group as compared to naïve/normal rats ($p \leq 0.001$) but repeated oral treatment of BIRM significantly inhibits CCI-induced upregulation of Iba-1 imuunoreactivity as compared to CCI-VC group (Figure 6.D)

Table 1: Effect of BIRM on paw withdrawal threshold in CCI-induced neuropathic pain in rats

Group	50% PWT of Ipsilateral Paws on Days				
	0	13	14	21	28
Normal Control	14.25±0.49	14.62±0.38	14.42±0.58	14.62±0.38	14.25±0.49
CCI-Vehicle control	14.62±0.38	3.00±0.54	3.11±0.53	4.30±1.28	2.63±0.28
CCI-BIRM	14.62±0.38	3.02±0.20	7.31±1.46*	8.34±1.29	11.31±1.24***
CCI-Gabapentin	14.62±0.38	3.11±0.28	12.98±0.90***	13.03±1.08***	12.84±1.02***

Values represented as Mean±SEM. PWT = paw withdrawal threshold, *p ≤ 0.05 , ***p ≤ 0.001 as compared to CCI-vehicle control group. Data analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Figure 2: Effect of BIRM treatment on mechanical sensitivity in rats after CCI on the sciatic nerve.
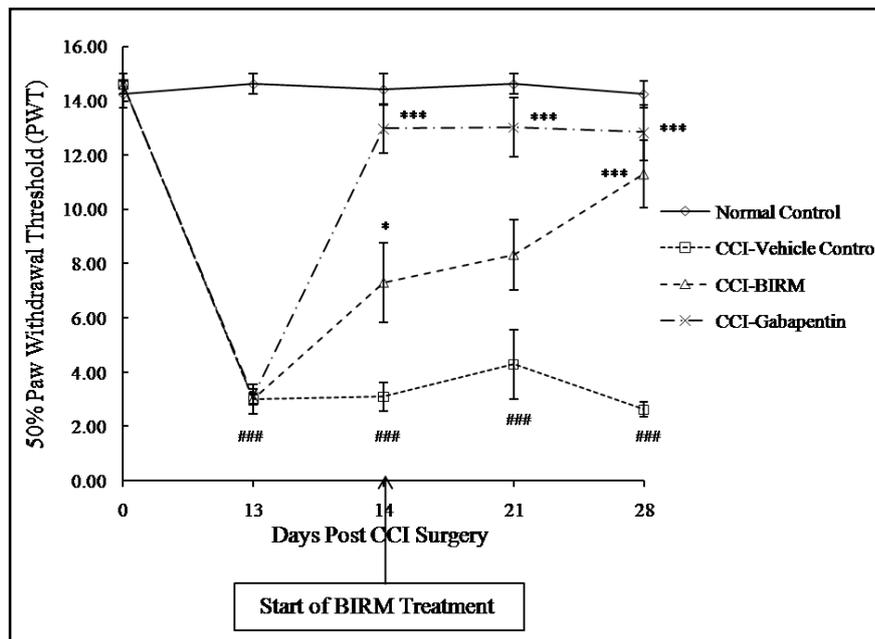
Response to tactile mechanical stimulus was measured in animals prior to surgery (day 0), prior to treatment (Day 13), and at 3 h post treatment on day 14, 21 and 28. Data presented are mean±SEM of median force (g) required to induce paw withdrawal in animals.
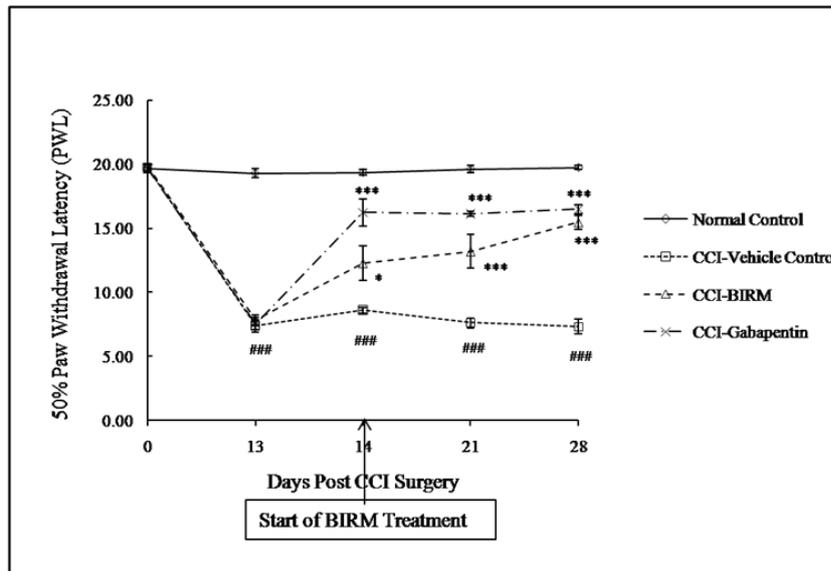
*p ≤ 0.05, ***p ≤ 0.001, as compared to CCI-vehicle control group. Significant changes were observed in CCI-vehicle control group as compared to Normal Control group (###p ≤ 0.001). Data analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Table 2: Effect of BIRM on paw withdrawal latency in CCI-induced neuropathic pain in rats

Group	50% PWL of Ipsilateral Paws on Days				
	0	13	14	21	28
Normal Control	19.70±0.20	19.29±0.36	19.35±0.22	19.62±0.27	19.75±0.17
CCI-Vehicle control	19.71±0.18	7.38±0.47	8.60±0.23	7.61±0.38	7.31±0.58
CCI-BIRM	19.84±0.11	7.84±0.40	12.27±1.37*	13.18±1.32***	15.47±0.55***
CCI-Gabapentin	19.63±0.26	7.55±0.47	16.23±1.05***	16.13±0.17***	16.47±0.35***

Values represented as Mean±SEM.sed by PWL= paw withdrawal latency, *p ≤ 0.05, ***p ≤ 0.001 as compared to CCI-vehicle control group. Data analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Figure 3: Effect of BIRM treatment on thermal hyperalgesia in rats after CCI on the sciatic nerve.

Response to thermal stimulus was measured in animals prior to surgery (day 0), prior to treatment (Day 13), and at 3 h post treatment on day 14, 21 and 28.

Data presented are mean±SEM of time (s) taken to respond (PWL) to thermal hyperalgesic stimulus. * p ≤ 0.05, ***p ≤ 0.001, as compared to CCI-vehicle control group. Significant changes were observed in CCI-vehicle control group as compared to Normal Control group (p ≤ 0.001). Data was analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Figure 4: Suppression of upregulated Iba-1 in the lumbar spinal cord tissue in CCI animals post BIRM treatment.
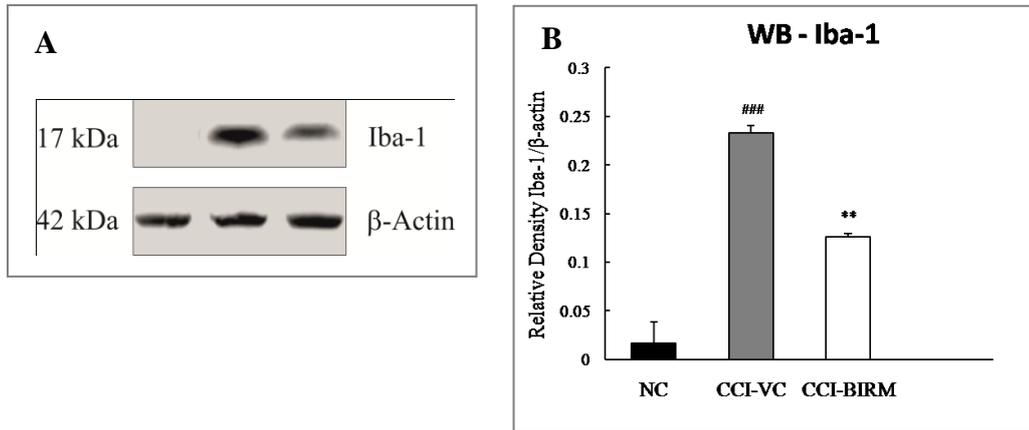
A significant increase in expression of Iba-1 protein was observed post CCI surgery. BIRM treatment shows visibly significant reduction in upregulation of Iba-1 expression (Figure 4A). Figure 4B depicts relative intensities of bands.

Data presented are mean±SEM of relative density Iba-1/β-actin. **$p \leq 0.01$ as compared to CCI-VC, [###]$p \leq 0.001$ as compared to Normal control group. Data analysed by One-way ANOVA followed by Tukey's multiple comparison test.

Table 3: Mean fold change in expression of target genes

Site	Target gene	Mean Δ ΔCt		Mean Fold change in Expression $2^{-\Delta\Delta Ct}$	
		CCI-VC	CCI-BIRM	CCI-VC	CCI-BIRM
Lumbar spinal cord	TNF-α	5.573	7.219	0.022±0.002	0.007±0.001** ↓
	IL-10	6.961	5.081	0.008±0.001	0.029±0.003** ↑
	COX-2	2.836	5.009	0.141±0.013	0.032± 0.006*** ↓

Data represented as Mean±SEM, ** $p \leq 0.01$, *** $p \leq 0.001$, Unpaired Student's t test
(Up arrow – Increased gene expression in CCI-BIRM compared to CCI-VC)
(Down arrow – Decreased gene expression in CCI-BIRM compared to CCI-VC)

Figure 5: Mean Fold change in mRNA levels of COX-2, TNF-α and IL-10 as compared to Normal control rats.
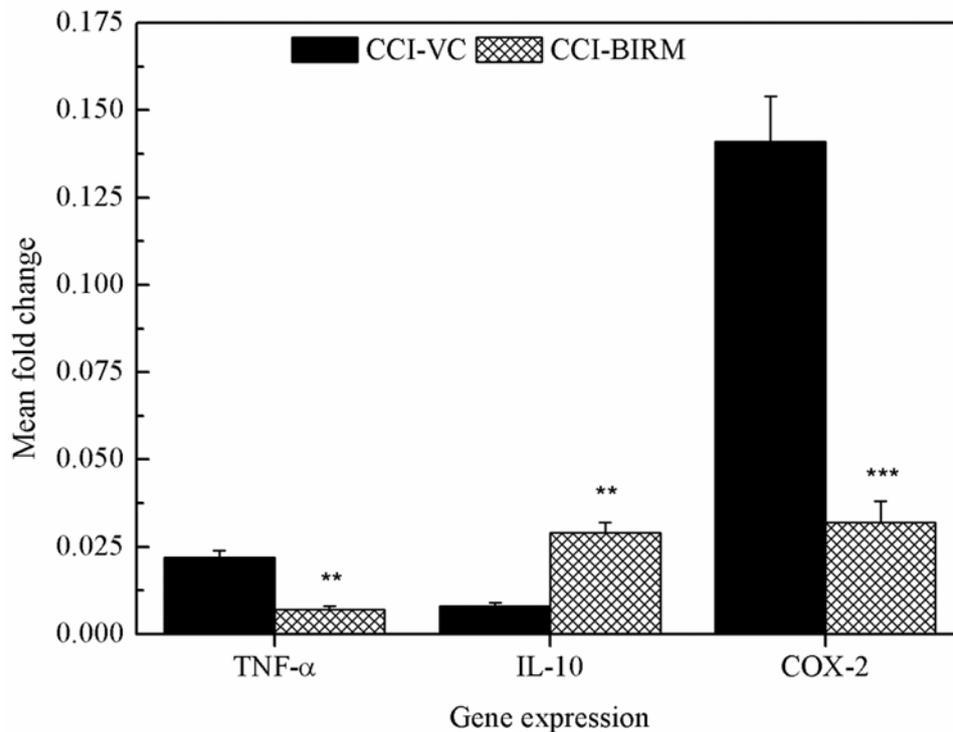
Tissue (dorsal lumbar spinal cord) collected 2 wks after administration of BIRM (4 ml/kg, p.o.) or vehicle in CCI rats. ** $p \leq 0.01$, *** $p \leq 0.001$ as compared to CCI-VC. Data analysed using unpaired Student's t test.

Figure 6: Immunostaining images of microglia cells labelled with Iba-1 (activated microglia cell marker) for the lumbar spinal cord sections.
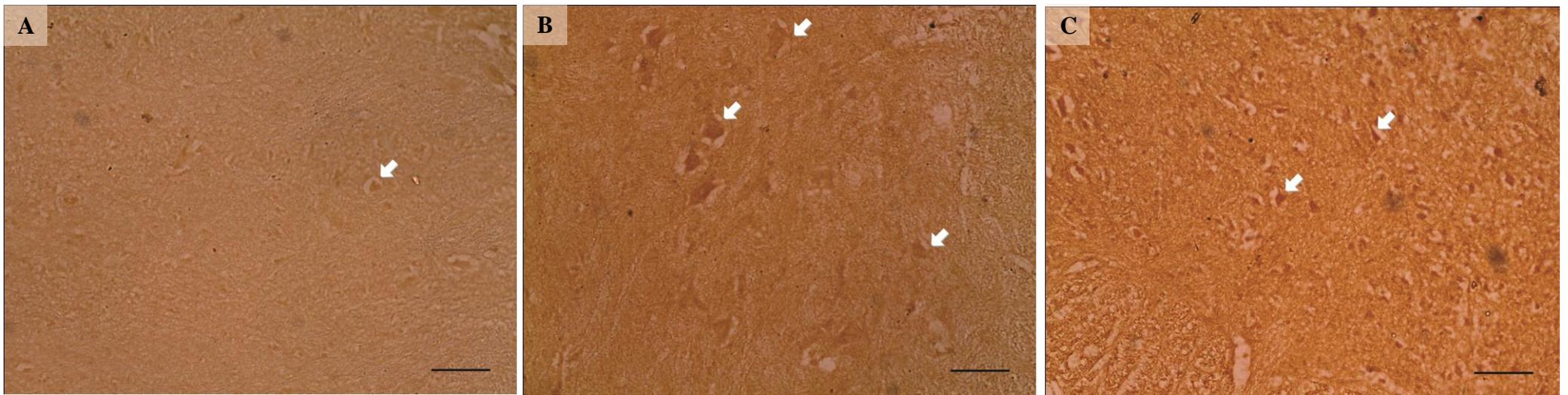
Figures 6A, 6B and 6C respectively show Iba-1 immunoreactivity in the lumbar spinal cord of naive/control rats, CCI-VC rats and CCI-BIRM rats on day 28 post CCI surgery. Basal levels of Iba-1 labelled microglia cells were observed in naive animal with resting morphology (A, arrow marked). Activated phenotype with marked cellular hypertrophy and retraction of processes as compared with control animals was observed in CCI rats (B). BIRM (4 ml/kg, p.o.) inhibits CCI-induced microglial activation to a large extent and reduces the activated phenotype of microglia cells and shift towards the resting stage was observed (C). Scale bar represents 100μm.

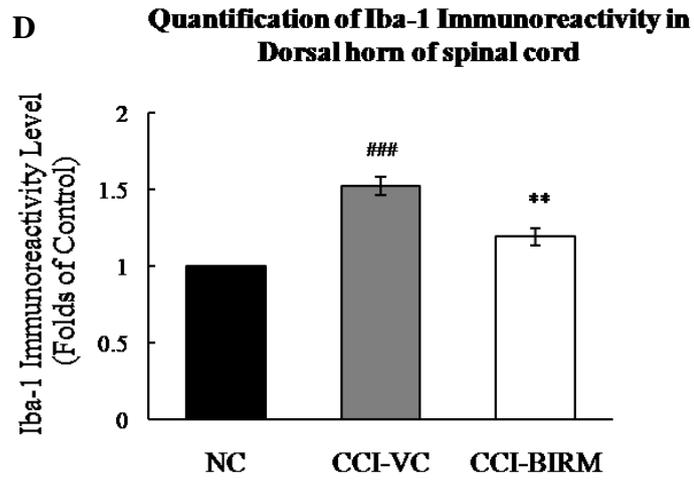**D**   **Quantification of Iba-1 Immunoreactivity in Dorsal horn of spinal cord**

Figure 6D shows Quantification of Iba-1 immunoreactivity indicating that BIRM significantly inhibited CCI-induced microglial activation in the dorsal horn of the spinal cord

Data was analysed using one way ANOVAfollowed by Tukey's multiple comparison test. *** p ≤ 0.001 as compared to NC, *p ≤ 0.05 as compared to NC, #p ≤ 0.001 as compared to CCI-VC.

DISCUSSION

The present study demonstrates that microglia cells are the useful tool for evaluating the effects of anti-neuroinflammatory effects of novel compounds. Repeated oral administration of BIRM, an aqueous extract of dried roots of *S. dulcamara*, has significantly inhibited thermal hyperalgesia and mechanical allodynia in animal model of CCI-induced neuropathic pain. The immunohistochemical results have shown the CCI-induced microglia activation, which is evident from their morphologies in CCI-VC group.The western blot results showed increased expression of Iba-1 protein in lumbar spinal cord in CCI induced neuropathic pain which further consolidates the immunohistochemical data.

Increased expression of Iba-1 protein under neuropathic condition indicates activation of microglia cells in the spinal cord. Repeated administration of BIRM orally, improved pathological conditions in animal model of neuropathic pain in the spinal cord by reducing the expression of Iba-1 protein and the proportion of activated microglia cells along with significant inhibition of neuropathic pain symptom, thermal hyperalgesia and mechanical allodynia.

Microglial cells are the resident immune cells of the central nervous system (CNS). They act as the main form of active immune defense in the CNS and upon getting activated following any insult to the nervous tissue, they become the main source of inflammatory mediators (e.g.: IL-1β, IL-6, TNF-α, PGE2, NO, BDNF etc) in the nervous system (Hung *et al.*, 2009). Microglia, once activated, gets engaged in phagocytosis and also participates in the adaptive immunity by presenting antigens to T cells. Apart from their role in inflammatory processes and regulation of cell survival, they are also capable of detecting specific aspects of normal and pathological levels of activity in brain, and its repercussions. Nerve injury induces extensive proliferation of spinal microglia and related gene expression. They become activated and adopt the immunological functions of the tissue following the damage (Mika, 2008). The increased presence of Iba-1 positive cells in L4-L5 region of spinal cord in present study following induction of neuropathic pain is in line with the earlier report on the L4-L5 spinal cord dorsal and ventral horn following sciatic nerve injury (Saxena *et al.*, 2007). Moreover, Patro *et al.*, (2010) have also reported activation of microglia and increased expression of Iba-1 in the proximity of the sensory and motor neurons in the L4-L5 spinal cord of the rats subjected to nerve injury. This data suggests importance of the role being assayed by Iba-1 protein in regulation of activated microglia functions. Using Iba-1 as microglia marker, Tawfik *et al.* (2007) and Romero-Sandoval *et al.* (2008) has also shown

the importance of role being played by microglia in the maintenance of neuropathic pain for longer duration. The microglial activation (presence of Iba-1 positive cells) in the L4-L5 region of spinal cord following induction of neuropathic pain through CCI reported in this article supports the above findings. Repeated administration of BIRM to CCI rats helps in restoring microglia cells to its resting stage from the activated stage (Figure 6C).

Further, the gene expression analysis of COX2 and pro-inflammatory cytokines (TNF-α) showed fold increase in their mRNA levels in lumbar spinal cord tissue of rats from CCI-induced vehicle control group as compared to normal control group. Repeated oral administration of BIRM not only inhibited the neuropathic pain symptom namely thermal hyperalgesia and mechanical allodynia but also prevented CCI-induced changes in spinal cord and significantly reduced fold increase of inflammatory mediators like COX-2 and TNF-α in lumbar spinal cord tissue. At the same time, we were able to observe fold increase in mRNA levels of anti-inflammatory cytokine (IL-10) in lumbar spinal cord tissue of CCI-BIRM treated rats.

It is well documented that COX exists in two isoforms - COX-1 and COX-2. COX-1 is constitutively expressed in most cells under physiological conditions whereas COX-2 is highly inducible in response to cytokines, growth factors, or other inflammatory stimuli and lasts for several months or even several years (Jaggi *et al.*, 2004; Zhu *et al.*, 2012). These enzymes catalyse the rate limiting steps of prostaglandin and thromboxane synthesis. Prostaglandins play a crucial role in nociceptive transmission at peripheral sites and in the spinal cord (Yamamoto and Nozaki-Taguchi, 2002; Jean *et al.*, 2008). The present gene expression studies show the mRNA levels of COX-2 in lumbar spinal cord of naïve rats. COX-2, being an inducible enzyme, increases in the peripheral and central nervous system post injury or inflammation (Seibert *et al.*, 1994; Zhao *et al.*, 2000) and plays an important role in neuropathology. Jean *et al.* (2009) observed overexpression of COX-2 in injured nerve in rats following CCI, partial sciatic nerve ligation, spinal nerve ligation and complete sciatic nerve transaction intervention. Supporting this observation, the present gene expression study shows a significant increase in COX-2 mRNA levels in the lumbar spinal cord in CCI rats as compared to normal/naïve rats (Figure 5A). Matsunaga *et al.* (2007) showed that inhibition of COX-2 by selective inhibitors attenuates hyperalgesia in neuropathic rats. This increase in COX-2 mRNA levels was inhibited by repeated oral treatment of BIRM in CCI-BIRM treated rats. These results suggest that BIRM produces an analgesic effect on neuropathy via inhibition of the expression of COX-2 mRNA levels in the spinal cord. The current results

support the earlier findings reported by Jaggi *et al.* (2004) demonstrating inhibitory effect of mother tincture *S. dulcamara* on PGE_2 production in *in vitro* conditions.

In addition, central neuroimmune activation and neuro-inflammation have also been postulated to mediate and/or modulate the pathogenesis of persistent pain states.

Pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), signaling proteins are uniquely powerful and have been associated with cell proliferation, differentiation and changes in gene expression and synthesis of matrix proteins important to cell growth and tissue repair (Wieseler-Frank *et al.*, 2005). They induce a long term alteration of synaptic transmission in the CNS and play a critical role in the development and maintenance of neuropathic pain (Sweitzer *et al.*, 2001a, b; Raghvendra *et al.*, 2002), but on the other hand they are also essential in fighting infection and responding to injuries. Each of these pro-inflammatory cytokines has been observed in spinal cord under pathological conditions implicating its role in pain facilitation. These cytokines activate neurons as wells as glia via specific receptors. In the CNS, the major contributors of cytokine release are glia. Microglia can produce cytokines on activation (Dong and Benveniste, 2001). Nerve injury or peripheral inflammation has been reported to activate glial cells and increase the pro-inflammatory cytokine levels in the CNS (DeLeo and Yezierski, 2001). Also as per the previous findings, TNF-α, IL-1 and /or IL-6 mRNA expression is elevated in spinal cord in response to peripheral nerve injury (Arruda *et al.*, 1998), spinal nerve injury (Winkelstein *et al.*, 2001), each of which elevates pain responses (hyperalgesia and allodynia). In line with this, the present study showed fold increase in mRNA levels of TNF- α in the lumbar spinal cord of rats following the sciatic nerve ligation (CCI) (Figure 5B). Repeated oral administration of BIRM to CCI-rats lowered the fold increase in mRNA levels of pro-inflammatory cytokine (TNF- α) (Figure 5B) and these could be due to its direct interaction with immune cells of the CNS. There are reports showing increase in TNF-α, IL-1 and/or IL-6 protein levels in spinal cord following peripheral injury (DeLeo, 1996; Winkelstein *et al.*, 2002).

IL-10, being a suppressor of macrophages, is considered as an anti-inflammatory cytokine. It potently down-regulates production and release of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 (Moore *et al.*, 1993). Although the precise functions of IL-10 in the CNS require further clarification, it is well known as an important negative regulator of pro-inflammatory gene expression. It can downregulate the expression of receptors for pro-inflammatory cytokines (Hamilton *et al.*, 1999; Sawada *et al.*, 1999). In our present study we

observed significant fold increase in IL-10 mRNA levels in the lumbar spinal cord CCI-rats treated with BIRM as compared to CCI-vehicle treated rats (Figure 5C). Ledeboer *et al.*, (2007) has reported that IL-10, when injected in a region of the spinal cord where activated glial cells were present, drastically reduced the pain symptoms in animal models of chronic pain. The latter record consolidates our current finding of BIRM induced heightened expression of IL-10 with concomitant reduction in the expression of TNF- α. This together with the observed reduction in the expression of COX-2 and the attended decline in prostanoid synthesis explain the reasons for the effective amelioration of neuropathic pain observed BIRM treated rats.

2. STREPTOZOTOCIN INDUCED DIABETIC PERIPHERAL NEUROPATHY IN RATS

Diabetes mellitus is an increasingly prevalent multiple organ system disorder with numerous devastating systemic effects. As per the survey data released by World Health Organization, it is estimated that nearly 336 million people worldwide will suffer from diabetes by the year 2030 (Wild *et al.*, 2004).

Neuropathy is one of the several manifestations resulting from diabetes and is considered to be a deleterious health effect and can be expected to occur in over half of all diabetic patients during their life span. Diabetic neuropathy encompasses a spectrum of clinical and subclincal conditions resulting in structural and functional alterations in peripheral as well as autonomic nerves and has a dramatic negative effect on the patient's daily quality of life and functions. Persistent or intermittent pain arising due to slight touch (allodynia), aberrant sensations (paraesthesia and dysesthesia), often accompanied with burning or tingling sensation and loss of sensations in extremities are the classic symptoms of diabetic neuropathies (mono/ polyneuropathies). Slowing down of nerve conduction in the large myelinated sensory and motor fibers occurring in early stage is considered as a prognostic indicator for peripheral neuropathy (Arezzo and Zotova, 2002). The early pathologic features of diabetic neuropathy include consequences of Schwann cell disruption (nodal widening, segmental demyelination, axonal degeneration and loss), basement membrane thickening and microvascular lesions followed by late stage features which include almost complete absence of large and small fibers in peripheral nerves corresponding to loss of both sensory and motor functions (Calcutt, 2004).

Genetic models and induced models (diet and drug induced) of diabetes in small laboratory animals are used to study the mechanisms of diabetic neuropathy. Although animal models of type 2 diabetes mellitus (genetic models) are the most representative to adult diabetic population, animal models of type 1 diabetes mellitus (drug and diet induced) are largely focused to study the mechanisms of diabetic neuropathy. Streptozotocin (STZ) induced diabetic model in rodents is commonly used because STZ has greater stability and it relatively lacks extra-pancreatic toxicity (Wattiez *et al.*, 2012). Behavioral responses to mechanical and thermal stimuli are the most widely studied parameters in animal studies of painful diabetic neuropathy and have clinical correlates.

Diabetic neuropathy, being a complicated pain, involves multiple mechanisms. Evidence generated through several clinical and experimental studies points towards functional changes at the spinal cord and neuroinflammation as an important factor in the process of central sensitization and its association with glial cells activation (Morgado *et al.*, 2010, Tumati *et al.*, 2012). Studies performed by Jung *et al.*, (2011) shows activation of spinal microglia in uncontrolled hyperglycaemic conditions followed by release of various proinflammatory cytokines like interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). These proinflammatory cytokines play an influential role in the induction of neuropathic pain and are thought to be contributing towards the pathogenesis of diabetic neuropathy. The cytokines and cyclooxygenase (COX) has been well correlated with pathophysiology of pain under pathophysiological parameters (Freshwater *et al.*, 2002). Going through these evidences suggests that counteracting the proinflammatory effects of microglia-produced cytokines may be one means of treating diabetic neuropathy.

The existing strategies for treatment of diabetic neuropathic pain is either based on pathogenetic concepts or based on symptoms Also, diabetic neuropathic pain being multifactorial in pathogenesis makes the clinical management a difficult task. Hence, alternate options are being sought after. Under these circumstances, BIRM (Biological Immune Response Modulator) seems to be promising herbal formulation. As stated earlier BIRM is an oral solution which has been formulated from extracts of Amazonian plant *Solanum dulcamara* by E. Cevallos-Arellano of Ecuador. This formulation is considered as a natural remedy for number of ailments that include AIDS, cancer etc. and is consumed as a dietary supplement by Ecuadorian native population (Cevallos, 1994). Earlier studies using the crude extract of *S. dulcamara* has shown inhibition of PGE_2 biosynthesis (Jaggi *et al.*, 2004). Based on the available data it was thought worth exploring the undiscovered benefits

of BIRM. Hence, a systematic study was envisaged wherein the microglia targeted anti-inflammatory property of BIRM in attenuating diabetic neuropathic pain was studied using STZ induced diabetic rat model.

MATERIAL AND METHODS

Animals and housing Conditions

Male Sprague Dawley rats (200-225g) were procured from CPCSEA and AAALAC approved vivarium facility at GVK Biosciences Pvt. Ltd., Hyderabad, India. They were allowed to acclimatize for a minimum duration of one week prior to induction of hyperglycemia. They were housed in groups of five in polypropylene cages under ambient conditions. Room temperature and humidity were maintained at 22-25°C and 65-70%, respectively. 12h light/dark cycle was maintained. Standard laboratory rodent diet and portable drinking water were provided *ad libitum*. Experimental protocols were approved by Institutional Animal Ethics Committee and all animal procedures were performed in accordance with the guidelines of CPCSEA. Possible efforts were attempted to minimize animal suffering, and to utilize minimum number of animals in this study.

Induction of diabetes

Diabetes was induced in 24 rats by administering single dose of streptozotocin (STZ) prepared in citrate buffer (pH 4.5, 0.1 M). The STZ was injected intraperitoneally at a dose of 50 mg/kg. Eight age-matched control rats were administered an equal volume of citrate buffer vehicle through intraperitoneal route. Forty-eight hours after the STZ injections, glucose concentration was measured from blood sample collected through tail vein using the Accu Chek Active (Roche Diagnostics, Germany). Rats having blood glucose concentration higher than 300 mg/dl were considered STZ-diabetic rats and were selected for study. Body weight and blood glucose were recorded once a week for the duration of the study.

About 8% morbidity rate was observed post-induction of diabetes. Around 25% animals were found refractory to STZ treatment and did not achieve the required blood glucose levels or exhibit allodynic behavior to mechanical stimulus.

Test Compound and Treatment Regimen

BIRM was a gift from BIRM Inc. (Quito, Ecuador). In the present study, BIRM samples from same lot number were used to avoid any variation in content.

Normal control and diabetic rats were randomly selected after assessment of mechanical allodynia and divided into three groups (N=8/group). BIRM dosing was initiated on day 21 (3 weeks) post-streptozotocin administration. BIRM was administered orally through stainless steel oral gavage needle. Animals from normal control (NC) and STZ administered vehicle control (STZ-VC) were administered distilled water throughout the study duration. Animals from BIRM-STZ group (STZ-BIRM) were administered BIRM daily at 4ml/kg dose volume for two weeks (day 21-day 35 post-streptozotocin administration). BIRM was clarified by centrifugation prior to usage as opined by Dandekar *et al.* (2003). Daily dose of 4ml/kg BIRM was selected based on dose range finding study using *in vivo* acute inflammatory model.

Neuropathic pain measurements

Mechanical Allodynia: It was assessed as described in the material and method part of CCI model at the beginning section of this chapter.

Thermal Hyperalgesia: It was assessed using Hargreaves method as described previously.

Cold Allodynia: Response to innocuous cold stimulation was measured by using acetone test (Choi *et al.*, 1994). The rats were placed in a plexiglass enclosure on a wire mash plate and allowed to acclimatize for 15-30 minutes prior to testing. Acetone drop (~50 µl) formed at the end of a 1ml BD syringe was gently applied to the plantar surface of the hind paws. Acetone was applied five times to each paw at intervals of 3-4 minutes. The brisk foot withdrawal response after the acetone application was considered as a positive response and the responses were graded to a four point scale: 0 = no response, 1= brisk withdrawal or flick of the paw, 2 = repeated flicking of the paw, 3 = repeated flicking of the hind paw and licking of the paw (Choi *et al.*, 1994; Xing *et al.*, 2007). The frequency of foot withdrawal was expressed as a percentage: (number of paw withdrawals/number of trials) x 100. The response scores were the average of the graded points in the five trials.

Sample Preparation

On day 35 post STZ administration (14 days post treatment), the rats (n=4 per group) from NC, STZ-VC and STZ-BIRM were euthanized and perfused immediately with 400-500 ml of cold phosphate buffered saline (0.01 M, pH -7.4) followed by 2% paraformaldehyde in 0.01 M phosphate buffer (pH-7.4) through the ascending aorta. Then their lumbar spinal cords (L4-L6 region) were quickly removed. From another set of four animals from each group, the

lumbar spinal cord tissue for RT-PCR and western blot analysis were immediately stored at -80°C until analysis.

Western blot: Expression of Iba-1 protein - microglia cell marker

Samples for western blot analysis were prepared as per the standard protocol detailed elsewhere.

RNA isolation and cDNA synthesis

Isolation of RNA and samples for cDNA were prepared as per the procedure described earlier.

Real Time- PCR (qPCR) analysis

Details of primers and procedures used are described in the first section of this chapter.

Immunohistochemistry

Procedures used for IHC are as described earlier. The pixel measurement was used for counting the density-slicing area in the image of the positive area of the dorsal horn of the spinal cord. Then, the relative changes in the staining density between NC, STZ-VC and STZ-BIRM were calculated using the following equation: (control pixels-treatment pixels)/(control pixels).

The criteria for resting and activated microglia were as described previously (Hains and Waxman, 2006). Briefly, the resting morphology is characterized by small compact somata bearing long, thin, ramified processes, and activated morphology is characterized by a process length less than the diameter of the soma compartment. Immunohistochemical data were expressed as the fold change compared with the normal control animals, which were considered to be 1. All samples from all groups were numbered randomly and blinded observation was carried out to prevent bias.

Statistical Analysis

Behavioral measures (PWL and PWT) were analyzed using one way ANOVA followed by Tukey's multiple comparison test. Data derived from cold allodynia was analysed using two way ANOVA followed by Bonferroni's post test Unpaired Student's t-test was used to analyse data generated from gene expression studies. $p \leq 0.05$ was considered statistically significant. For ease of reading, the basic statistical values are shown in the text while the more extensive statistical information can be found in the figure legends.

RESULTS

Effects of BIRM on body weight and blood glucose levels

The initial body weights (prior to administration of streptozotocin) were similar in all animals selected for the study. After streptozotocin injection (50 mg/kg, i.p.), rats developed hyperglycemia within one week in greater than 90% of animals. At 3 weeks post STZ administration, diabetic rats exhibited a significant decrease in body weight and increase in blood glucose levels as compared to their age matched non-diabetic control animals (NC). However, significant improvement in blood glucose levels was observed in diabetic animals treated with BIRM (STZ-BIRM) on day 35. Diabetic animals treated with BIRM presented decrease in body weight loss as compared to diabetic vehicle control group (STZ-VC) (Table 4).

Effects of BIRM on diabetic neuropathic pain related behavioral measurements

Animals were subjected to behavioral measurements prior to STZ injection (Day 0) and at regular intervals post STZ injection (day 20, 21, 28 and 35 post STZ treatment). After induction of diabetes, rats developed allodynia and hyperalgesia. Average of 50% paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) measured in both paws among the groups was considered for analysis.

Mechanical Allodynia

Allodynia - parameter showing significant change in threshold for withdrawal from non-painful stimuli, develops quite early but it is not uniform in all diabetic animals. In our experiment, allodynia developed in 18 animals out of 24 animals. These 18 animals were randomly divided into two groups (STZ-VC and STZ-BIRM) and were used for further analysis.

A significant reduction in 50% paw withdrawal threshold (PWT) was observed 3 weeks post STZ injection as compared to age matched normal control animals (1.94 ± 0.31, STZ *vs* 14.51 ± 0.25, NC, $p \leq 0.001$). Repeated administration of BIRM showed improvement in paw withdrawal threshold through average protection of 24% and 63% at 3 hr post BIRM administration on day 28 and day 35, respectively. This increase in 50% PWT was significantly higher than STZ-vehicle control group ($p \leq 0.001$) as analysed by one way ANOVA followed by Tukey's multiple comparison posttest (Table 5 and Figure 7).

Thermal Hyperalgesia

The threshold for thermal hyperalgesia was significantly reduced by 3 weeks after STZ administration as compared to age matched control animals (7.85 ± 0.43, STZ *vs* 17.52 ± 0.17, NC, p ≤ 0.001). BIRM, when administered daily (4 ml/kg, p.o.) for a duration of 2 weeks shows significant increase in paw withdrawal latency to the thermal stimulus by average protection of 33% and 57% on day 28 and day 35, respectively. This increase in 50% PWL was significantly higher than STZ-VC (p ≤ 0.001) as analysed by one way ANOVA followed by Tukey's multiple comparison post-test (Table 6 and Figure 8).

Cold Allodynia

Animals that received STZ injection, developed significant allodynic response to the acetone on day 20 post STZ injection (p ≤ 0.01 as compared to NC). Allodynic response to acetone test included quick withdrawal of the paw, stumping or flicking of the paw, prolonged withdrawal or repeated flicking of the paw often accompanied with licking of ventral side of the exposed paw. The response to frequent acetone exposure (total of 5 trials/hind paw) is expressed as response frequency (Figure 9A) or response scores (Figure 9C). This response was found to be maximum in STZ treated rats when measured on day 20 post STZ injection. It was maintained at same level in the STZ-VC animals where there was no intervention of any drug treatment. However, animals given STZ injection but treated with BIRM from day 21 post STZ injection, showed significant reduction in response to acetone exposure (p ≤ 0.001 as compared to STZ-VC). Figure 9B represents summary of BIRM effect on response frequency from day 21 to day 35. The response frequency (%) was 78% and 30% as observed on day 28 and day 35, respectively. Fig 9D represents summary of BIRM effect on response scores. Repeated treatment of BIRM shows significant reduction in response score as compared to animals of STZ-VC group (p ≤ 0.001). Data was analysed using two way ANOVA followed by Bonferroni's multiple comparison test.

Effect of BIRM on Iba 1 protein expression in diabetic neuropathic pain condition

Iba-1 protein, being marker of activated microglia cells was studied through western blot. Spinal Iba-1 protein level when quantified using western blot, was found to be increased in STZ-VC group on day 35 post STZ injection as compared to animals of NC group. Repeated administration of BIRM for 14 days was found to be effective in reducing spinal Iba-1 protein level as compared to STZ-VC group. This observation indicates neuroprotective effect of BIRM on repeated administration in neuropathic pain condition arising due to diabetes (Figure 10).

Effect of BIRM on spinal expression of COX-2, TNF-α and IL-10 in diabetic neuropathic condition

Mean fold increase was observed in mRNA levels of TNF-α (1.399 times) and COX-2 (1.441 times) in lumbar tissue (L4-L5) in STZ-VC animals as compared to normal control (NC) animals. Mean fold change of 0.361 times was observed in mRNA levels of IL10 in animals of STZ-VC group. But treatment with BIRM was able to bring significant (p ≤ 0.01) reduction in mean fold change in mRNA levels of TNF-α (0.081 times) (p ≤ 0.001) and COX-2 (0.174 times) in lumbar tissue (L4-L5) in STZ-BIRM animals as compared to STZ-VC group. Similarly, significant increase in fold change in mRNA levels of IL-10 (1.302 times) (p ≤ 0.05) in CCI-BIRM group was observed as compared to CCI-VC group. Data was analysed using unpaired Student's t test. (Table 7 and Figure 11)

Effect of BIRM on microglia cells in Spinal cord under Diabetic neuropathic pain condition

Morphology of Spinal microglia cells was studied using Iba-1 antibody, which is very often used to selectively label activated microglia cells through immunolabelling technique. Very few Iba-1 positive cells were visible in lumbar spinal cord (L4-L5) sections from NC group. Compared to NC, there was significant increase in Iba-1 positive cells in spinal cord sections of STZ-VC and STZ-BIRM groups. Microglia cells resembling morphology of resting stage with small soma and long processes were observed in spinal cord sections of NC group (Figure 12A). As compared to NC group, distinct morphology of microglia cells with large soma and smaller processes was observed in sections of STZ-VC and STZ-BIRM groups (Fig 12B and 12C). Shift from resting stage to activated stage of microglia was observed in STZ treated animals, but repeated treatment with BIRM for duration of 14 days contributes in reversal of this shift towards the resting stage (Figure 12C). Alterations in morphology of microglia cells in STZ treated animals were mainly found to be present in median part of the dorsal horn. These findings are in agreement with observation made by Tsuda *et al.*, 2008.

In addition to this, when Iba-1 immunoreactivity in lumbar dorsal horn was quantified, we found that there was significant increase in Iba-1 positive cells in STZ treated groups – STZ-VC and STZ-BIRM as compared to normal rats (NC) (Figure 12D).

Table 4: Effect of BIRM on Mean body weight and blood glucose levels

Group	Body Weight (g)		Blood Glucose (mg/dl)	
	Day 20	Day 35	Day 20	Day 35
NC	248±2.49	273±3.19	101±1.65	100±1.59
STZ - VC	216±1.57*	204±2.10*	380±5.20*	430±16.93*
STZ - BIRM	212±2.12*	223±1.57*#	378±8.70*	206±9.82*#

Values represent are mean ± SEM, STZ = Streptozotocin, *p ≤ 0.001 vs. NC, #p ≤ 0.001 vs. STZ-VC, One way ANOVA followed by Tukey's multiple comparison test

Table 5: Effect of BIRM on paw withdrawal threshold in diabetic peripheral neuropathic rats

Group	50% PWT of Ipsilateral pwas on Days				
	0	20	21	28	35
NC	14.02±0.41	14.51±0.25	13.87±0.25	14.06±0.49	14.04±0.52
STZ - VC	13.43±0.39	1.94±0.25###	1.52±0.15###	0.87±0.06###	0.67±0.02###
STZ - BIRM	14.34±0.33	1.99±0.34	1.86±0.22	4.29±0.39***	9.69±0.50***

Data represented as Mean±SEM. ***p ≤ 0.001 as compared to STZ-VC group. ###p ≤ 0.001 as compared to normal control group.

Data analysed by One way ANOVA followed by Tukey's multiple comparison test.

Figure 7: Attenuation of Mechanical allodynia by BIRM in diabetes induced neuropathy model in rats
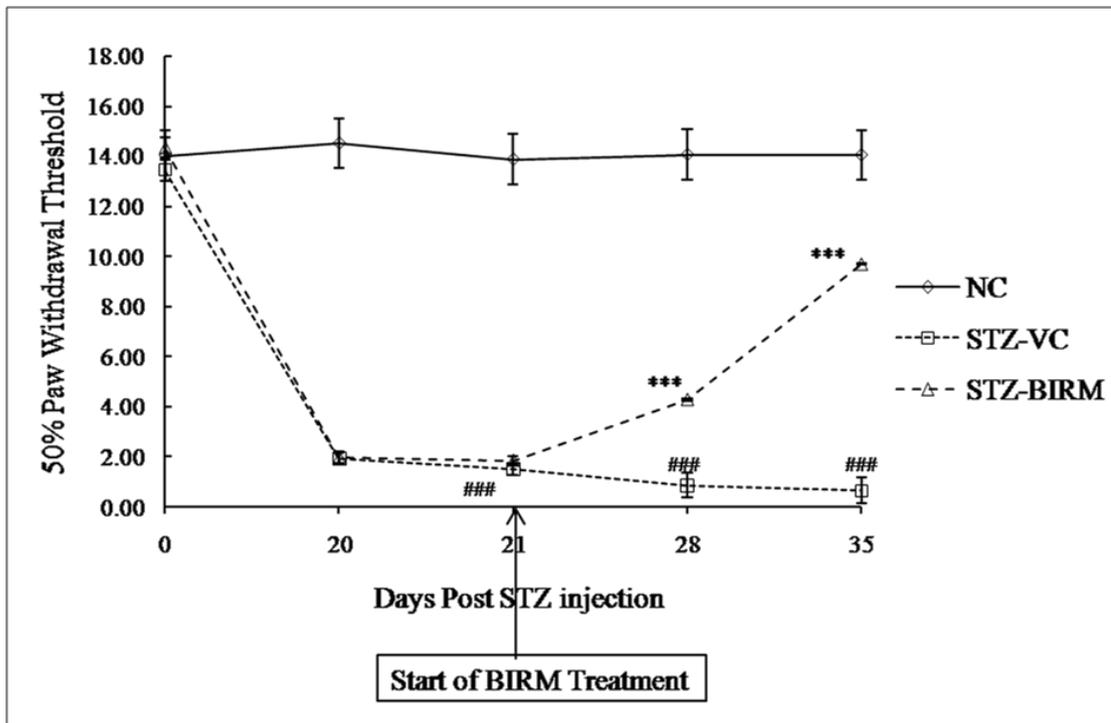
STZ treated rats gradually developed allodynia to mechanical stimulus post STZ injection. Repeated treatment of BIRM improves the PWT on day 28 and day 35. BIRM treatment was initiated on day 21 and continued till day 35.

p ≤ 0.001 as compared to NC, *** p ≤ 0.001 as compared to STZ - VC, Data analyzed by One way ANOVA followed by Tukey's multiple comparison test.

Table 6: Effect of BIRM on paw withdrawal latency in diabetic peripheral neuropathic rats

Group	50% PWL of Ipsilateral pwas on Days				
	0	20	21	28	35
NC	17.61±0.19	17.52±0.17	17.30±0.23	17.30±0.18	17.44±0.23
STZ - VC	16.98±0.26	7.79±0.47###	7.24±0.20###	6.75±0.20###	6.62±0.35###
STZ - BIRM	17.13±0.29	7.90±0.38	7.57±0.22	11.09±0.29***	14.27±0.33***

Data represented as Mean±SEM. ***$p \leq 0.001$ as compared to STZ-VC group. ###$p \leq 0.001$ as compared to normal control group.

Data analysed by One way ANOVA followed by Tukey's multiple comparison test.

Figure 8: Attenuation of thermal hyperalgesia by BIRM in Diabetes induced Neuroapthy model in rats
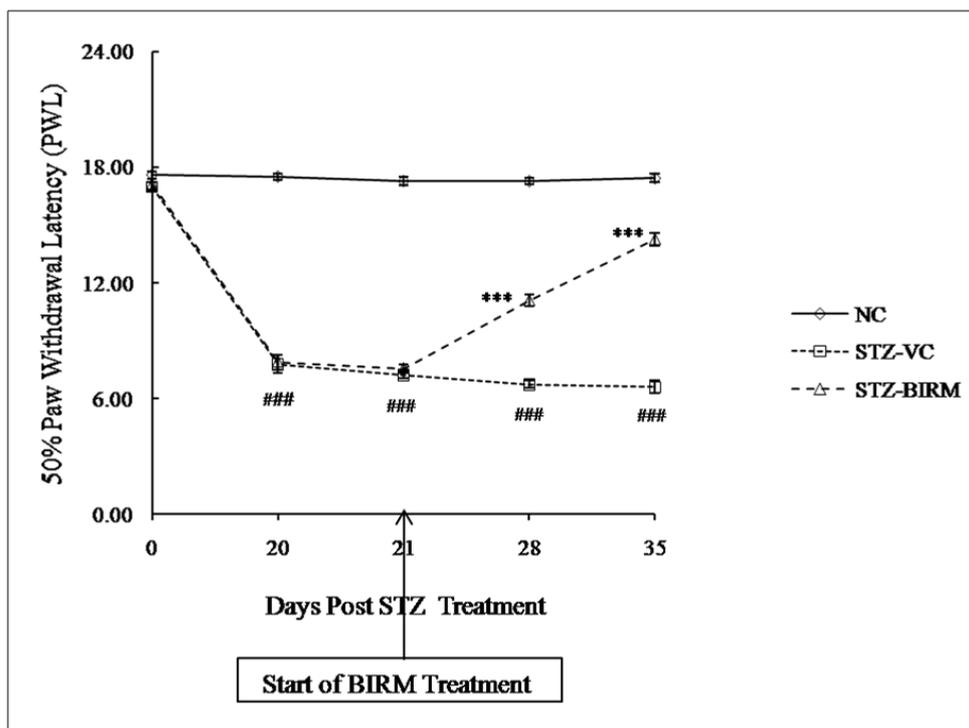
STZ treated animals developed significant hyperalgesia to thermal stimulus on day 20 post STZ injection. Repeated treatment of BIRM shows improved in paw withdrawal latency to thermal stimulus over a course of treatment time.

*** $p \leq 0.001$ as compared to STZ-VC, ### $p \leq 0.001$ as compared to NC, Data analyzed by One way ANOVA followed by Tukey's multiple comparison test.

Figure 9: Attenuation of cold allodynic response by BIRM in diabetes induced neuropathic model in rats.
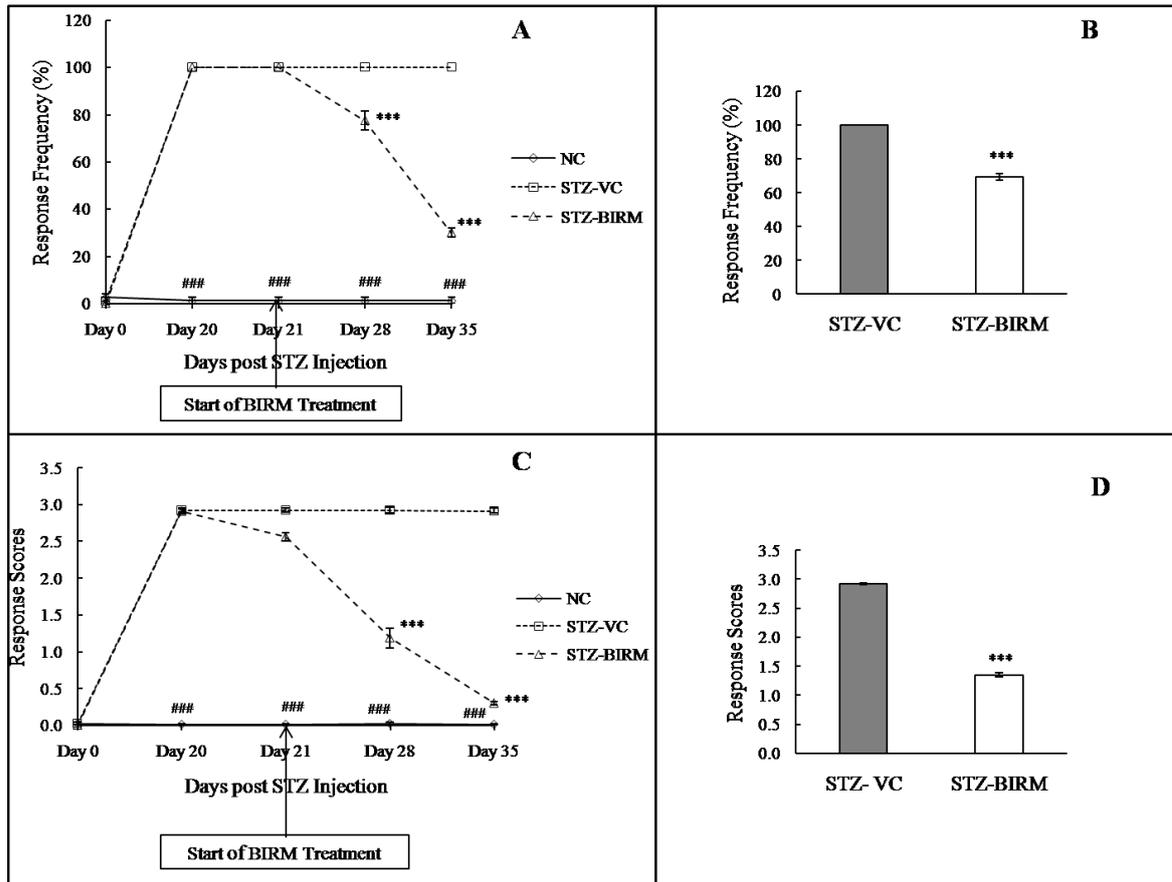
A. Cold allodynic response frequency (%) to acetone test in hind paws of the diabetic neuropathic rats (n=8) on days post STZ injection. B. Summary of cold allodynic response frequency after the administration of BIRM (from day 21-day 35 post STZ injection). C. Cold allydonic response represented as response scores to acetone test in diabetic neuropathic rats post STZ injection. D. Summary of cold allodynic response scores after the administration of BIRM (from day 21-day 35 post STZ injection).

BIRM treatment was initiated on day 21 and continued till day 35. Error bars indicate Mean ± SEM. (p ≤ 0.05), ***p ≤ 0.001 as compared to STZ-VC, ### p ≤ 0.001 as compared to NC. Two way ANOVA followed by Bonferroni posttests

Figure 10: Down-regulation of Spinal Iba-1 levels by BIRM in Diabetic Neuropathy
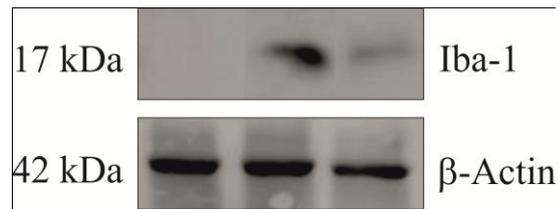
Spinal Iba-1 levels increases on day 35 post STZ injection. Repeated administration of BIRM shows its effect through down-regulating Iba-1 protein in diabetic neuropathic animals.

Table 7: Effect of BIRM on spinal expression of COX-2, TNF-α and IL-10 in diabetic neuropathic condition

Site	Target genes	Mean Δ ΔCt		Mean Fold change in Expression $2^{-\Delta\Delta Ct*}$	
		STZ-VC	STZ-BIRM	STZ-VC	STZ-BIRM
Lumbar spinal cord	TNF-α	-0.470	3.6248	1.399±0.133	0.081±0.002 *** ↓
	IL-10	2.997	-0.378	0.361±0.102	1.302±0.047 * ↑
	COX-2	-0.504	2.269	1.441±0.208	0.174±0.010 ** ↓

$* \ p \le 0.05, ** \ p \le 0.01 , *** \ p \le 0.001$ as compared to STZ-VC. Unpaired Student's t test

(Up arrow − Increased gene expression in STZ-BIRM group as compared to STZ-VC group)
(Down arrow − Decreased gene expression in STZ-BIRM group as compared to STZ-VC group)

Figure 11: Mean Fold change in mRNA levels of COX-2, TNF-α and IL-10 as compared to Normal control rats.
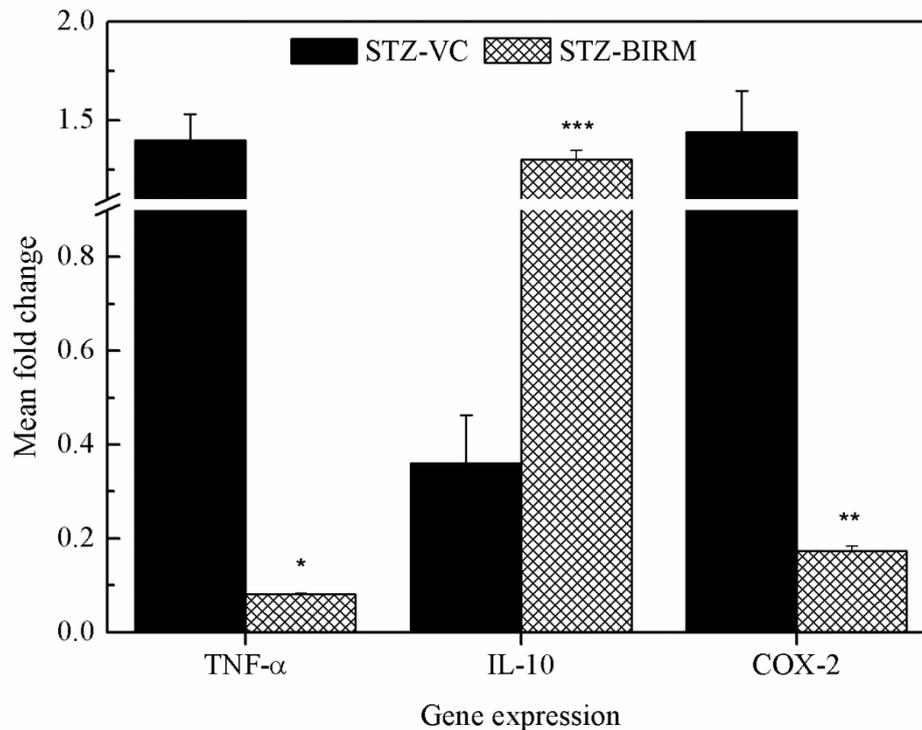
Tissue (dorsal lumbar spinal cord) collected 2 wks after administration of BIRM (4 ml/kg, p.o.) or vehicle in STZ treated rats. $*p \le 0.05, **p \le 0.01, ***p \le 0.001$ as compared to STZ-VC. Data analysed using unpaired Student's t test.

Figure 12: Immunostaining images of microglia cells labelled with Iba-1 (activated microglia cell marker) for the lumbar spinal cord sections.
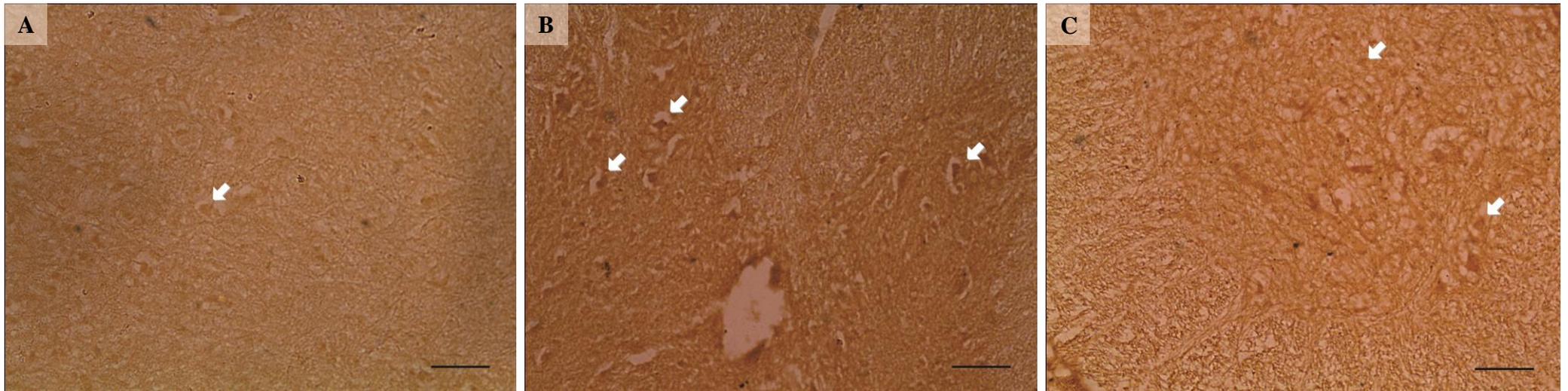
Figures 12A, 12B and 12C respectively show Iba-1 immunoreactivity in the lumbar spinal cord of naive/control rats, STZ-VC rats and STZ-BIRM rats on day 35 post STZ administration. Basal levels of Iba-1 labelled microglia cells were observed in naive animal with resting morphology (A, arrow marked). Activated phenotype with marked cellular hypertrophy and retraction of processes as compared with control animals was observed in STZ treated rats (B). BIRM (4 ml/kg, p.o.) inhibits diabetic neuropathy-induced microglial activation to a large extent and reduces the activated phenotype of microglia cells and shift towards the resting stage was observed (C). Scale bar represents 100μm.

D **Quantification of Iba-1 Immunoreactivity in dorsal horn of spinal cord**
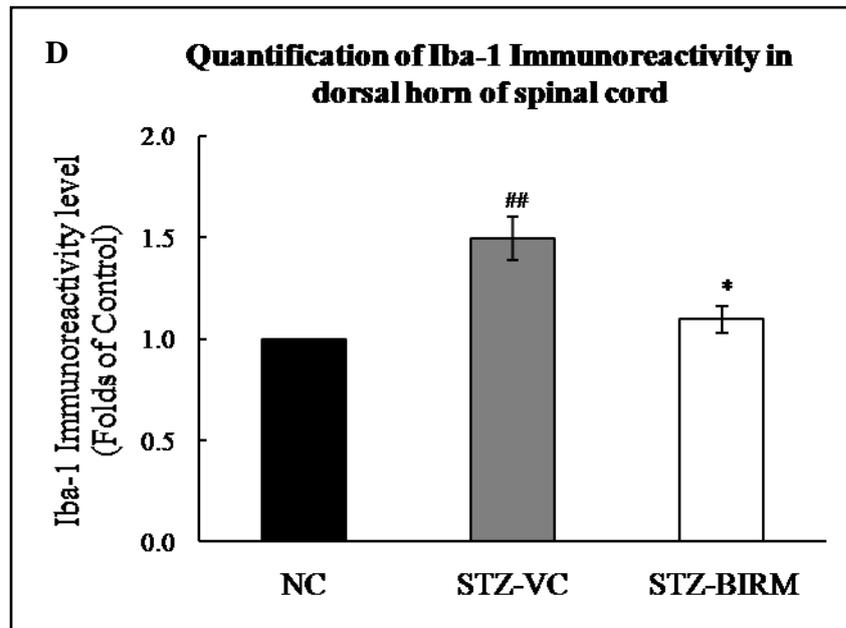
Figure 12D shows quantification of Iba-1 immunoreactivity indicating that BIRM significantly inhibited CCI-induced microglial activation in the dorsal horn of the spinal cord. Data was analysed using one way ANOVAfollowed by Tukey's multiple comparison test. ## $p \leq 0.01$ as compared to NC, *$p \leq 0.05$ as compared to STZ-VC.

DISCUSSION

Neuropathic pain being one of the major complications of diabetes mellitus, STZ-induced diabetes rat model was used to study chronic neuropathic pain. It develops behavioral abnormalities like allodynia and hyperalgesia during the course of disease which is believed to be having correlation with pain symptoms under clinical conditions. The aim of our study was to study the neuro-protective effect of BIRM in diabetes induced neuropathy and study the role of microglia and cytokines in maintaining the pathological environment.

Development of diabetic condition was confirmed by hyperglycemic state accompanied with loss of body weight in animals treated with STZ. Progression of diabetes gradually led to development of diabetic neuropathy, which was confirmed by changes in nociception. Changes in nociception were evident by decreased paw withdrawal latency (thermal hyperalgesia), decreased paw withdrawal threshold to mechanical (von Frey) and increased response to chemical allodynic stimulation (acetone test). This behavioral observation is in confirmation with earlier findings by Courteix et al. (1998), indicating that hyperglycemia induced by diabetes in rats alters pain sensitivity by producing both allodynia and hyperalgesia. However, there are reports showing that early development of thermal hyperalgesia is independent of glycemic levels of animals (Bishnoi et al., 2011), pointing towards the fact that factors other than hyperglycemia may be triggering early thermal hyperalgesia (Pabbidi et al., 2008; Romanovsky et al., 2010). Repeated oral administration of BIRM for over a period of 14 days does improve blood glucose levels and shows reduction in body weight loss in animals treated with STZ. It also exhibits improvement in threshold level to mechanical and chemical allodynia and increases paw withdrawal latency to thermal hyperalgesia as compared to STZ treated animals (STZ-VC group). These results indicate towards the blood glucose lowering potential of BIRM in diabetic conditions.

Earlier studies have shown involvement of Cyclooxygenase (COX) system in transmission of pain stimuli in STZ induced diabetic neuropathy (Bujalska et al., 2008). Also there are reports indicating infiltration of inflammatory cells in response to any damage to nervous system which in turn leads to production and secretion of various cytokines, growth factors or inflammatory mediators such as bradykinins, serotonin, prostaglandins (PGs) or nitric oxide (Moalem and Tracey, 2006; Bujalska and Gumulka, 2008). In addition to this, various animal models of neuropathic pain have been performed to study involvement of inflammatory mediators (such as PGs) in mechanisms underlying the development of neuropathic pain (Syriatowicz et al., 1999; Hefferan et al., 2003; Ma and Eisenach, 2003; Zhu and Eisenach,

2003; Durrenberger *et al.*, 2006; O'Rielly and Loomis, 2006). Ma and Eisenach (2003) have also shown in their studies that PG(s) synthesized by COX-1 and COX-2 may play a role in sensitization of nociceptors in neuropathic pain. In line with this above observation, in our present study, significant fold increase in mRNA levels of COX-2 in lumbar spinal cord was observed in STZ treated animals (STZ-VC) as compared to normal rats (NC), indicating its role in generating neuropathic pain. After repeated administration of BIRM (4 ml/kg) daily for duration of two weeks in STZ treated animals (STZ-BIRM), significantly reduced fold increase in mRNA levels of COX-2 as compared to STZ-VC group. These results support the earlier findings of Jaggi *et al.* (2004) demonstrating inhibitory effect of mother tincture *S. dulcamara* on PGE_2 production by hampering cyclooxygenase activity in *in vitro* conditions.

Till sometime back it was understood that altered neuronal activity in the primary sensory neurons and spinal cord neurons are responsible for hyperalgesia observed in neuropathic pain but observations made by Watkins *et al.* (1997) indicates role of glial cells in the pathogenesis of pain through the release of neuroactive factors including prostanoids and cytokines. Studies conducted using various animal models also emphasize microglia getting activated in spinal cord under circumstances like cancer pain (Mantyh *et al.*, 2002), nerve injury (Winkelstein *et al.*, 2001) and diabetes induced neuropathic pain (Tsuda *et al.*, 2003; Daulhac *et al.*, 2006; Wodarski *et al.*, 2009; Morgado *et al.*, 2011; Graeber and Christie, 2012; Kim *et al.*, 2012,) and microglia inhibition through glia inhibitors like minocycline helping in amelioration of neuropathic pain (Raghvendra *et al.*, 2003). Supporting the above observations, we noticed significant up-regulation of Iba-1 protein, a marker of activated microglia cells in lumbar spinal cord tissue of STZ treated animals (STZ-VC) as compared to normal rats (NC). Similarly, through immmunohistochemical studies, we could observe significant increase in number of activated Spinal microglia cells (identified through their altered morphology). BIRM, the drug of our interest, when administered daily for 14 days showed reduction in Iba-1 protein expression in lumbar spinal cord as compared to STZ-VC. With respect to morphological aspects of microglia cells, we observed a shift from activated to resting state of microglia cells in BIRM treated group (STZ-BIRM).

Microglia cells being the active immune defense system in the central nervous system, are the main source of inflammatory mediators (e.g.: IL-1β, IL-6, TNF-α, PGE_2, NO, BDNF etc) in the nervous system when subjected to injury or insult (Hung *et al.*, 2009). This means that activation of microglia cells also leads to increased synthesis and secretion of inflammatory mediators. There are studies indicating IL-1β, IL-6 and TNF-α playing a critical role in

inducing hyperactivity of dorsal horn neurons (central sensitization) ultimately leading to pain hypersensitivity (Kawasaki *et al.*, 2008; Ren and Torres, 2009). These inflammatory mediators released through microglia has the potential to modulate spinal cord synaptic transmission, leading to increased excitability of dorsal horns neurons, partially through suppression of inhibitory synaptic transmission (Wen *et al.*, 2011). These pro-inflammatory cytokines are site-specifically produced and released from dorsal spinal cord in response to agents capable of inducing hyperalgesia or allodynia and are thought to act in a way where they are capable of causing their own preservative release (Milligan *et al.*, 2001). In our present study, we observed significant fold increase in mRNA levels of TNF-α in dorsal spinal cord in fifth week post STZ treatment (STZ-VC) as compared to normal control animals (NC).There are several studies showing implications of spinal proinflammatory cytokines in pain modulation by blocking or disrupting their actions, which in turn block/improve the exaggerated pain state (Milligan *et al.*, 2000; Sweitzer *et al.*, 2001a, b; Watkins *et al.*, 2001a, b). Similarly, repeated treatment with BIRM for 14 days was able to significantly reduce the fold increase in mRNA levels of TNF-α in dorsal spinal cord as compared to STZ-VC group. This was reflected in terms of improved paw withdrawal threshold to mechanical and chemical stimuli and increased paw withdrawal latency to thermal stimuli. This observation also indicates direct interaction of BIRM with immune cells in the central nervous system. Also supporting our observations, studies by DeLeo *et al.* (1996) and Winkelstein and DeLeo. (2002) have reported increased expression levels of TNF-α, IL-1 and/or IL-6 in spinal cord in animal models of neuropathic pain.

It is known fact the creation of imbalance in the pro-inflammatory and anti-inflammatory cytokines is also one of the reasons for pain development. Anti-inflammatory cytokines such as IL-1α, IL-4 or IL-10 are believed to inhibit development of neuropathic pain by playing crucial role in nocicpetion (Zychowska *et al.*, 2013). Moore *et al.* (1995) and Milligan *et al.* (2005) have reported that IL-10 being the powerful anti-inflammatory cytokine has the potentials of suppressing the production and release of pro-inflammatory cytokines (TNF- α, IL-1, IL-6). Under the pathological conditions, reduction in anti-inflammatory cytokine levels is observed and any intervention with drug increases the levels of anti-inflammatory cytokines and improves the pathological condition. In our study we observed that there was treatment with BIRM for 14 days had impact on mRNA levels of IL-10 thus increasing its mRNA levels in spinal cord as compared to STZ-VC. However, observations made by Rojewska *et al.* (2014) are in contrast with our observations, where significant upregulation

of mRNA IL-10 was observed in spinal cord and dorsal root ganglion (DRG) in neuropathic rats (CCI-induced neuropathic pain) as compared to normal rats but treatment with Minocycline reduced the mRNA IL-10 levels in spinal cord as well as in DRG.

CONCLUSION

In the present study, we have characterized two animal models of peripheral neuropathic pain namely chronic constriction injury (CCI) and STZ-induced diabetic peripheral neuropathy.
We could observe activation of microglia cells and overexpression of pro-inflammatory mediators (COX-2 and TNF- α) in dorsal horn. Thermal hyperalgesia and allodynia to mechanical and thermal stimulus were also observed. Repeated treatment with BIRM was able to downregulate Iba-1 expression along with reduction in TNF-α and COX-2 levels thus restoring the neuro-immune balance. It also attenuated pain behavior such as hyperalgesia and allodynia.

Further, BIRM is able to modulate microglia activation and ameliorate the pathological condition by increased threshold to allodynic stimuli, it can be concluded that activated microglia play a crucial role in creating and maintaining exaggerated pain state. In the light of the study conducted herein it could be concluded that BIRM has the potential to improve the pathological conditions of peripheral nerve injury due to metabolic disorder or even surgical intervention and would emerge as a potential therapeutic agent in the treatment of neuropathic pain subsequent to clinical validations.