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AMAZONIAN PLANT EXTRACT BIRM REVERSES CHRONIC NEUROPATHIC PAIN IN RAT SCIATIC NERVE CHRONIC CONSTRICTION INJURY MODEL

Ravalji, M.[1], Buch, P.[1], Uggini, G. K.[1], Awasthi, A.[2], Cevallos-Arellano, E.[3]; Balakrishnan, S.[1*]

[1]Department of Zoology, The M. S. University of Baroda, Vadodara 390 002, Gujarat, India
[2]Division of Biology, GVK Biosciences Pvt. Ltd., Hyderabad 500 076, Telangana, India
[3]BIRM Inc, Quito, Ecuador

[*]suved9@hotmail.com

Abstract

Neuropathic pain condition remains poorly managed by currently available therapeutics. There is therefore a dire need for development of efficacious therapeutics with minimal side effects. BIRM (Biological Immune Response Modulator), an extract of Amazonian plant *Solanum dulcamara*, consumed as a dietary supplement by natives in Ecuador, is considered as a natural remedy for a number of ailments (AIDS and Cancer, among others). The aim of the current study was to test the efficacy of BIRM in *in vivo* neuropathic pain model to elucidate its anti-neuroinflammatory potential. Rats subjected to chronic constriction injury (CCI) were divided into CCI-control, CCI-Gabapentin and CCI-BIRM groups along with a normal control group. BIRM was administered orally (4 ml/kg, daily) to animals of CCI-BIRM group from day 14 post surgery till day 28. Repeated oral administration of BIRM inhibited CCI-induced mechanical allodynia and thermal hyperalgesia. It also inhibited CCI-induced activation of microglial cells and upregulation of COX-2 and TNF-α in the dorsal horn of the lumbar spinal cord. These data indicate that the marketed formulation BIRM, has anti-neuroinflammatory and anti-nociceptive properties in neuropathic rats and can serve as an adjuvant to standard therapy or as a stand-alone therapeutic agent for the treatment of neuropathic pain disorders.

Keywords: Biological Immune Response Modulator (BIRM), neuropathy, neuroinflammation, microglia cells.

Introduction

Neuropathic pain is a chronic pain condition and is heterogeneous in nature. It is considered to arise from damage to nerves due to tumors, diabetic neuropathy, herpes zoster, complex regional pain syndrome, AIDS, sclerosis multiplex, hypoxia or stroke [1] and occurs worldwide. 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% [2]. Symptoms of neuropathic pain are often severely debilitating such as allodynia, hyperalgesia, spontaneous pain, as well as behavioral disabilities.

Several animal models mimicking peripheral nerve injury have been developed to study neuropathic pain. Most widely used animal models are chronic constriction injury (CCI) of sciatic nerve [3], partial ligation of the sciatic nerve (PNL) [4] and ligation of one or more of the spinal nerves (SNL). 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 [5]. Emerging lines of evidence have revealed that changes also occur in spinal microglia, the immune cells of the central nervous system [6]. 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.

Till date, Neuropathic pain remains a poorly managed pathological condition by currently available therapeutics. Current treatments available for neuropathic pain indicate general insensitivity to non-steroidal anti-inflammatory drugs and relative resistance to opioids. These treatments have untoward side effects when given at higher doses to obtain adequate analgesia [7]. Researchers around the globe are looking for alternate treatment which can offer adequate analgesia devoid of severe side effects. Under these circumstances, BIRM (Biological Immune Response Modulator) seems to be promising herbal formulation. BIRM is an oral solution which has been 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 (AIDS, cancer) and is consumed as a dietary supplement by Ecuadorian native population [8].

Earlier studies using BIRM have shown anti-metastasis properties in *in vivo* prostate cancer model [9] and inhibition of PGE_2 production by COX1 and COX2 [10]. Recently, studies performed using BIRM in preclinical animal models of pain and inflammation exhibit its analgesic and anti-inflammatory properties [11]. Based on these data available, we found it good enough to explore undiscovered benefits of BIRM and study it systematically for assessing its anti-nociceptive effect in neuropathic pain conditions.

Methods

Animals and housing conditions

Male Wistar rats (180-210 g) 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. 12 h light/dark cycle was maintained. Standard laboratory rodent diet and portable 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. Experimental protocols were approved by IAEC (Institutional Animal Ethics Committee) of GVK Biosciences Pvt. Ltd. according to CPCSEA (Committee for the purpose of Control and Supervision of Experiments of Animals), India [1125/PO/c/CPCSEA/019(2012)] All animal procedures were performed in accordance with guidelines of CPCSEA.

Chronic constriction injury

The method described by Bennett and Xie [2] was generally followed. Rats were anesthetized with gaseous anesthesia 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 & Johnson) were tied loosely around it with an interval of about 1 mm

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.

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 *S. 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 10,000 g prior to usage as described by Dandekar et al. [9]. Gabapentin (Sigma Aldrich, USA) was used as a standard reference. 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). The commercially available formulation was directly administered without further dilution at 4 ml/kg b.wt. BIRM when administered orally at 4 ml/kg dose level was found to be well tolerated and found no observable systemic toxicity in rodents.

Experiment was performed in two sets (n=4 per group in each set). Data obtained from both sets was collated and is represented hereby as one experiment. Surgically operated CCI rats were randomly selected after assessment of mechanical allodynia and divided into four groups (n=8 per group). Drug testing was initiated on day 14 post surgery. Animals from Normal Control (NC) and CCI operated Control (VC-CCI) were orally administered distilled water throughout the study duration. Animals from BIRM –CCI operated group (BIRM-CCI) were administered BIRM daily at 4 ml/kg dose volume (day 14-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. The 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 [12].

Thermal Hyperalgesia

Thermal response was determined by measuring hind paw withdrawal latency of affected paw employing Hargreaves' plantar test.

Sample Preparation

On day 28 post surgery (14 days post treatment), the rats from NC, CCI-VC and CCI-BIRM were deeply anesthetized with isoflurane 5% 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 – microglia cell marker

Protein was resolved on 15% polyacrylamide gel followed by transfer on to nitrocellulose membrane. Immunoprobing of Iba-1 protein was by Anti-Iba-1 (AbCam, ab5076) used at 1:1000 dilution. For staining, Horseradish peroxidase coupled with ECL detection reagent (GE Healthcare-Amersham, USA) was used. Anti-β-actin was used as the loading control antibody. Densitometric analysis was carried out using Alpha Ease FC software, version 4.0.034 (Alpha Innotech, USA) and results were normalized to loading control.

Real time PCR

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 (Invitrogen) to remove any contaminating DNA. Total RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). All cDNA samples were stored at -80°C until real-time PCR (qPCR) was performed.

The primers for TNF-α, IL10, COX-2 and GAPDH were F: 5 '– GATGGGCTGTACCTTATCTACTCCCAGG-3',R 5'-CCTTAGGGCAAGGGCTCTTGATGGC; 5' – TAAGGGTTACTTGGGTTGCCAAGCC-3', reverse 5'– GCAGCTGTATCCAGAGGGTCTTCAGC - 3'; 5' – CAGTATCAGAACCGCATTGCCTCTG -3', reverse 5'–

GTGAGCAAGTCCGTGTTCAAGGAGG - 3'; 5' – CAAGGTCATCCATGACAACTTTGGC - 3', reverse 5'– CAAGGTCATCCATGACAACTTTGGC - 3', respectively. 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 Step-one Real Time PCR machine (Applied Biosystems). 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. 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 $2^{-\Delta(\Delta Ct)}$.

Immunohistochemistry

Spinal cord tissues collected after transcardial perfusion were washed with phosphate buffer and cryopreserved in sucrose 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 using goat polyclonal anti-Iba1 antibody (1:300; Abcam, overnight incubation at 4°C). The ABC-DAB system was used for immuostaining. From each animal's spinal cord, four to five sections within the L4-L5 region were included in the analysis. 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. Colour intensity was 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 [13]. All samples from all groups were numbered randomly and blinded observation was carried out to prevent bias.

Statistical Analysis

One way ANOVA 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

The effect of BIRM on CCI -induced changes in behavioral and neuropathic pain measurements

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 ($p>0.05$) in animals subjected to CCI surgery (CCI-VC, CCI-BIRM and CCI-Gabapentin) after characterization of CCI on day 13 (Table 1 and 2; Figure 1 and 2) .

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 ($P \leq 0.001$). Repeated oral treatment with BIRM (4 ml/kg) as a single dose daily for 14 days showed a mean 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 ($p \leq 0.05$) but not on day 21 as analysed by One Way ANOVA followed by Tukey's multiple comparison test. BIRM showed mean protection of 70% on day 28. This increase in 50% PWT was significantly higher than CCI - vehicle control group ($p \leq 0.001$) as analysed by One Way ANOVA followed by Tukey's multiple comparison test (Table 1; Figure 1). Treatment with Gabapentin at 30 mg/kg showed a mean protection of 82%-83% at all assessment time i.e., on day 14, 21 and 28. The increase in 50% PWT was significantly higher ($P \leq 0.001$) than CCI - vehicle control group as analyzed by One Way ANOVA followed by Tukey's multiple comparison test (Table 1; Figure 1).

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 ($p \leq 0.001$). But treatment with BIRM (4 ml/kg/day) showed a significant increase in PWLs at 3h post administration

on day 14 ($p \leq 0.05$), day 21 ($p \leq 0.001$) and day 28 ($p \leq 0.001$). 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 (Figure 2).

Treatment with Gabapentin showed an average increase in PWLs at 3 hr post administration on 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 2).

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 ($p<0.001$). Significant reduction in Iba-1 protein level was observed in the CCI group treated with BIRM ($p\leq0.01$) (Figure 3). 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 IL-10 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-BIRM 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. Results were analysed using Unpaired Student's t test (Figure 4).

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 5A) as compared to higher number of Iba-1 positive cells in CCI-VC and CCI-BIRM sections (Figure 5B and 5C). The morphology of microglia cells in normal control sections exhibited the resting type shape to some extent, which has small compact somata bearing long, thin, ramified processes (Figure 5A, 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 5C). 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 ($p\leq0.001$) but repeated oral treatment of BIRM significantly inhibits CCI-induced upregulation of Iba-1 imuunoreactivity as compared to CCI-VC group (Figure 5D).

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 a plant of the species *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 supports 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, PGE_2, NO, BDNF etc) in the nervous system [14].

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 [1]. 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 [15]. Moreover Patro *et al*. [16] 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*. [17] and Romero-Sandoval *et al*. [18] 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 5C). Further, the gene expression analysis of COX-2 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 [10, 19]. 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 [20, 21]. The present gene expression studies show the mRNA levels of COX-2 in lumbar spinal cord of naïve rats (Figure 4). COX-2, being an inducible enzyme, increases in the peripheral and central nervous system post injury or inflammation [22,23] and plays an important role in neuropathology. Jean *et al*. [24] 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 4). Matsunaga *et al*. [25] 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*. [10] demonstrating inhibitory effect of mother tincture *Solanum dulcamara* on PGE_2 production via COX-1 and COX-2 *in vitro*. 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 [26]. 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 [27, 28], 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 [29]. Nerve injury or peripheral inflammation has been reported to activate glial cells and increase the pro-inflammatory cytokine levels in the CNS [30].

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 [31], spinal nerve injury [32], 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 4). Repeated oral administration of BIRM to CCI-rats lowered the fold increase in mRNA levels of pro-inflammatory cytokine (TNF-α) (Figure 4) 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 nerve injury [32, 33].

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 [34]. 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 down-regulate the expression of receptors for pro-inflammatory cytokines [35, 36]. In our present study we observed significant fold increase in IL-10 mRNA levels in the lumbar spinal cord of CCI-rats treated with BIRM as compared to CCI-vehicle treated rats (Figure 4). Ledeboer *et al*. [37] have 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 in BIRM treated rats.

In summary, our study with BIRM shows inhibition of microglia activation in the CNS and downregulates pro-inflammatory cytokines and COX-2. As demonstrated in this study, BIRM attenuates the development of hyperalgesia and allodynia in the rat model of neuropathic pain. Overall, this study not only demonstrates the effectiveness of BIRM in improving pathological conditions of nerve injury induced neuropathic pain but also showed the important role played by microglia in regulating the induction of a chronic pain state induced by peripheral nerve ligation.

Based on the results obtained using BIRM as a therapeutic agent in neuropathic pain model at preclinical stage, BIRM has potential to improve pain condition or reduce the disease progression as a standalone therapeutic or adjuvant to standard therapy.

Conflict of Interest

There is no conflict of interest.

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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.

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.

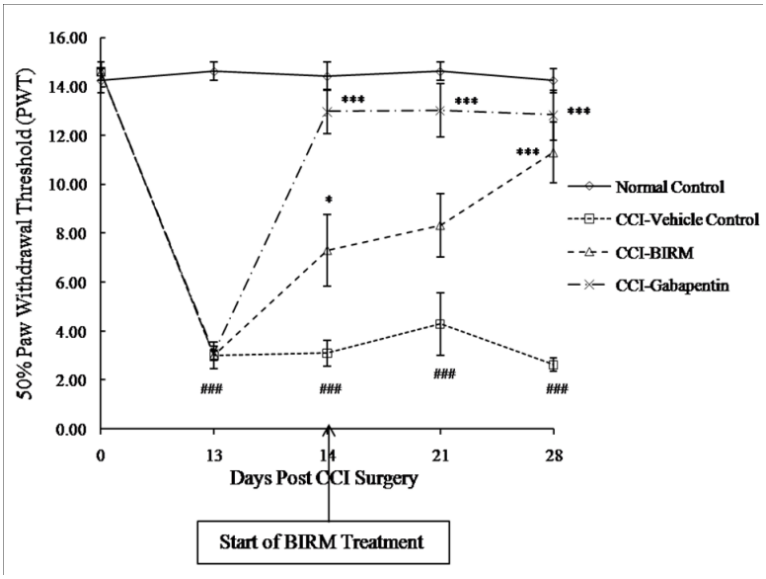

Figure 1. Effect of BIRM treatment on mechanical sensitivity in rats after CCI on the sciatic nerve. Response to tactile mechanical stimulus was measured in animals prior to surgery (day 0), prior to treatment (Day 13), and at 3 h post treatment on day 14, 21 and 28. Data presented are mean ± SEM of 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.

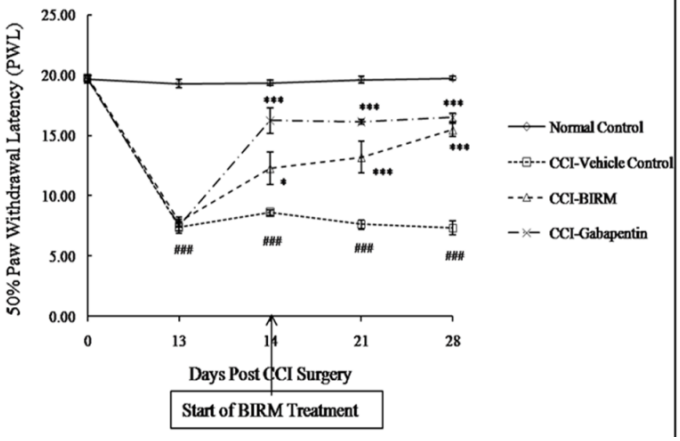

Figure 2. Effect of BIRM treatment on thermal hyperalgesia in rats after CCI on the sciatic nerve. Response to thermal stimulus was measured in animals prior to surgery (day 0), prior to treatment (Day 13), and at 3 h post treatment on day 14, 21 and 28. Data presented are mean ± SEM of time (s) taken 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.

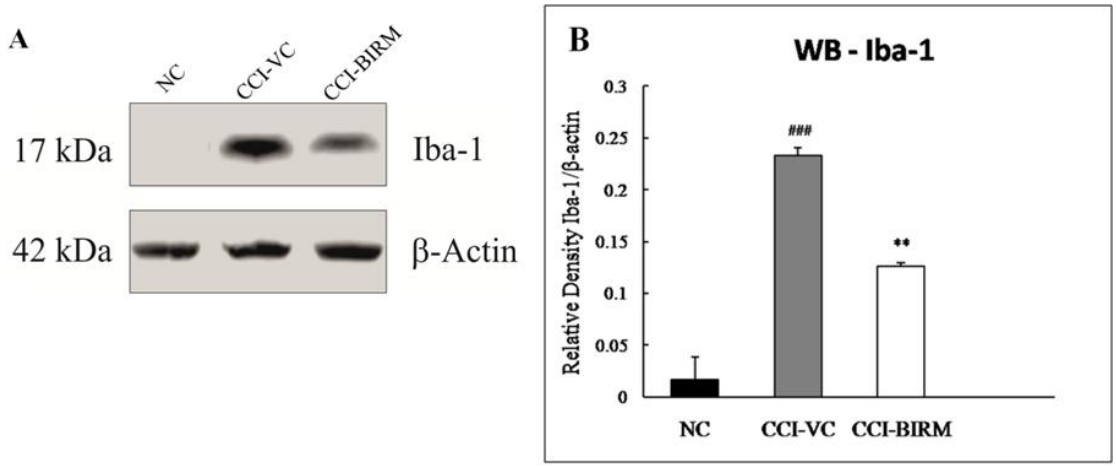

Figure 3. Suppression of upregulated Iba-1 in the lumbar spinal cord tissue in CCI animals post BIRM treatment. A significant increase in expression of Iba-1 protein was observed post CCI surgery. BIRM treatment shows visibly significant reduction in upregulation of Iba-1 expression (Figure 3A). Figure 3B depicts relative intensities of bands. Data presented are mean±SEM of relative density Iba-1/β-actin. **p ≤ 0.01 as compared to CCI-VC, ###p ≤ 0.001 as compared to Normal control group. Data analysed by One-way ANOVA followed by Tukey's multiple comparison test.

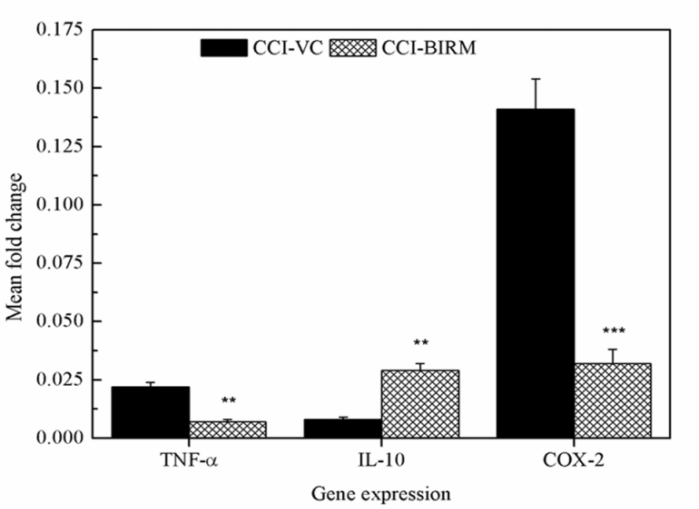

Figure 4. Mean Fold change in mRNA levels of COX-2, TNF-α and IL-10 as compared to Normal control rats. Tissue (dorsal lumbar spinal cord) collected 2 wks after administration of BIRM (4 ml/kg, p.o.) or vehicle in CCI rats.
**p ≤ 0.01, ***p ≤ 0.001 as compared to CCI-VC. Data analysed using unpaired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

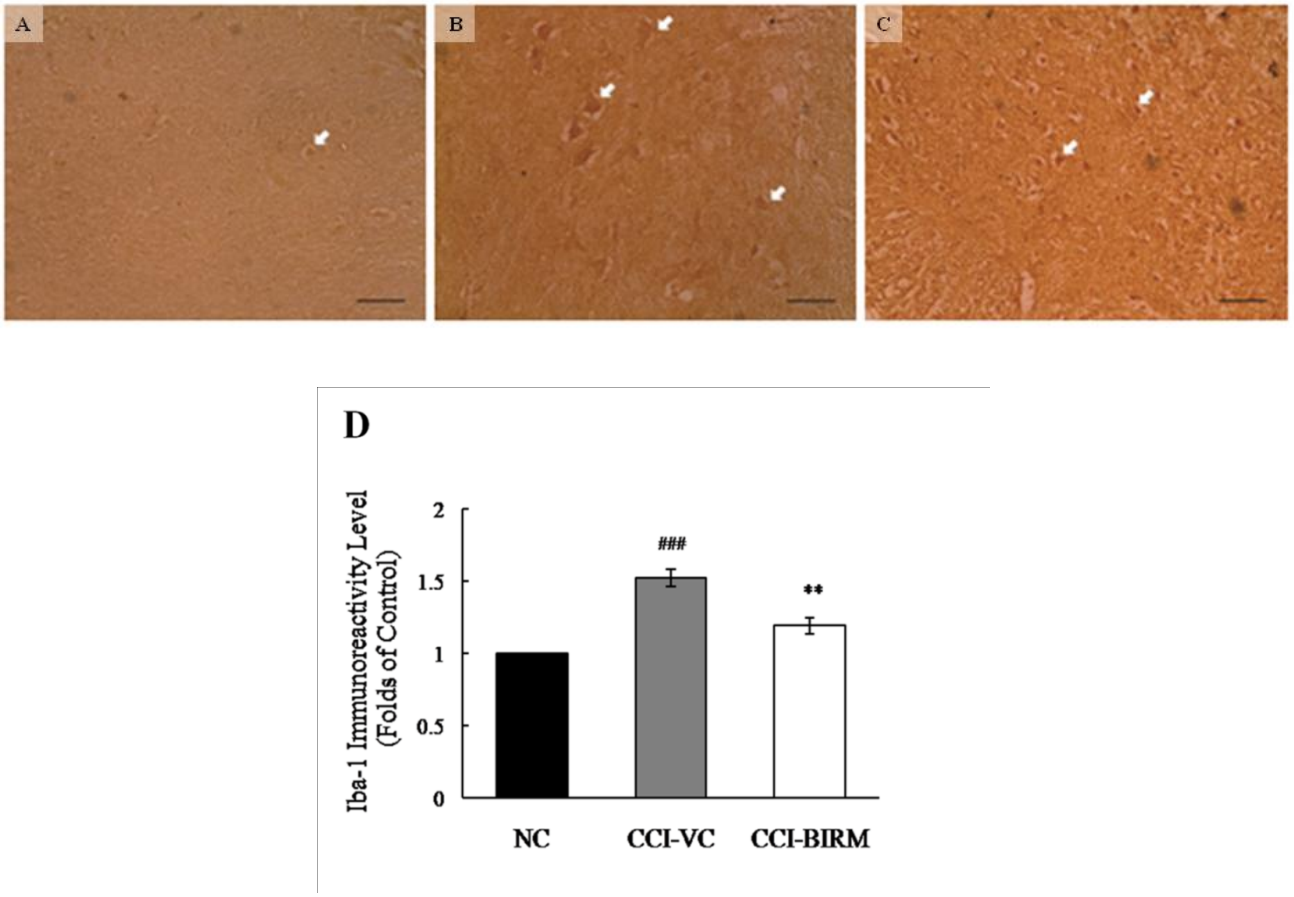

Figure 5. Immunostaining images of microglia cells labelled with Iba-1 (activated microglia cell marker) for the lumbar spinal cord sections.

Figures 5A, 5B and 5C 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.

Figure 5D 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 ANOVA, followed by Tukey's multiple comparison test. ###$p \leq 0.001$ as compared to NC, **$p \leq 0.01$ as compared to CCI-VC.