Pre-emptive PPAR-γ Activation Abolishes Development of Nerve Injury-induced Behavioral Hypersensitivity: Elucidating Underlying Mechanisms

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Neuropathic pain is a chronic incapacitating painful condition for which there is no effective treatment. The peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear transcription factors that play key roles in modulating immune and inflammatory responses. The antinociceptive properties of PPAR-γ activation on development of neuropathic pain are not fully known.
Objective: To determine the role of PPAR-γ activation on the development of neuropathic pain following chronic constriction injury and to elucidate underlying mechanisms.
Methodology: Neuropathy was induced by chronic constriction injury of sciatic nerve in rats. Cold allodynia and thermal hyperalgesia were assessed and the markers of inflammation and nitroso-oxidative stress were estimated.

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Results: Pre-emptive administration of pioglitazone, a PPAR-γ agonist (3, 10 or 30 mg/kg, i.p. 1 hr before surgery and continued once daily for 2 weeks) dose-dependently attenuated paw withdrawal latency to cold (allodynia) and thermal (hyperalgesia) stimuli. Pioglitazone significantly reduced elevated TBARS, protein carbonylation, nitrite levels and markedly restored depleted GSH, and reduction in activities of SOD and catalase in injured nerves. Further, pioglitazone markedly reduced plasma extravasation and levels of pro-inflammatory cytokines TNF-α and IL-1β following nerve injury. Moreover, pioglitazone did not alter the locomotor activity. Pretreatment with PPAR-γ antagonist BADGE (30 mg/kg, i.p.) blocked the beneficial effects of pioglitazone. Essentially, pioglitazone promoted the long-lasing recovery and also prevented the development of neuropathic pain even after treatment termination.

Conclusion: Pioglitazone, a PPAR-γ agonist receptor-dependently abolished the development of traumatic neuropathic pain and exerted long-lasting antinociceptive effects through reducing nitroso-oxidative stress and inflammation. Our findings strongly suggest that pre-emptive activation of PPAR-γ prevented or at least delayed the development of nerve injury-induced pain hypersensitivity.

1. INTRODUCTION

Peripheral nerve injury often results in neuropathic pain including both hyperalgesia and allodynia. Several lines of evidence suggest that nerve injury is associated with local inflammation and plasma extravasation [1]. Moreover, neuroinflammation also occurs after nerve injury and involves release of various chemical mediators including pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, nitric oxide (NO) and prostaglandins (PGs) which can originate locally or from cells that infiltrate the site of inflammation [2,3]. These cells initiate inflammatory and immune response in which firstly the mast cells are activated followed by sequential recruitment and activation of neutrophils and macrophages [3]. Recently, investigators have placed emphasis on the role of activated non-neuronal cells, particularly microglia and astroglial cells, of the spinal cord in the exaggerated pain states [4]. Upon activation, these inflammatory cells release pro-inflammatory and pronociceptive mediators including reactive oxygen species (ROS), reactive nitrogen species (RNS) [5].

Accumulating evidence indicates ROS play an important role in the peripheral and central sensitization of neuropathic pain [5,6]. It has been reported that these inflammatory mediators modulate the pain processing by directly causing the neuronal excitability or indirectly through the release of NO and PGs which play an important role in the development of neuropathic pain [7]. In this context, current drug therapies do not control safely and effectively this type of severe chronic pain state and even widely used drugs often produce multiple side effects, particularly severe CNS toxicity [8]. Therefore, there is an urgent need for the development of therapeutic agents capable of blocking abnormal pain sensation without impairing normal abilities.

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that play key roles in the regulation of immune and inflammatory response [9]. Upon ligand binding, PPARs regulate transcription by forming heterodimeric complex with retinoid X receptor (RXR) that binds to particular sequence on DNA, peroxisome proliferator response element (PPRE), so as to induce or repress the transcription of particular target gene [10]. PPAR-α, PPAR-β/δ and PPAR-γ are three structurally homologous isotypes found in various species which display wide range of distinct effects on metabolism, cellular proliferation, differentiation, and the immune response [9,10]. It is well known that nuclear transcription factors, PPAR-γ, are expressed on neutrophils, monocytes/macrophages, microglia, and neurons [11]. Furthermore, PPAR-γ activate and regulate macrophage differentiation and down regulate pro-inflammatory mediators in activated macrophages and microglia, mainly by inhibiting transcription of nuclear factor (NF)-κB dependent inflammatory genes [10,11].

Thiazolidinediones (TZDs), are insulin sensitizers, include rosiglitazone, pioglitazone, englitazone, and ciglitazone are potent synthetic agonists of PPAR-γ which are commonly prescribed for the treatment of type-2 diabetes.

Several studies emphasized the effects of PPAR-γ agonists to prevent acute and chronic nociception and inflammation [12,13]. Accumulating data indicates that PPAR-γ agonists have also shown protection against inflammatory diseases of various etiologies include multiple sclerosis, Alzheimer's disease, Parkinson's disease, and spinal cord injury [14]. Furthermore, several studies also reported that PPAR-γ activation alleviates oxidative stress and improves antioxidant defenses following spinal cord injury and inflammation [15,16]. These data suggest that PPAR-γ could be a novel pharmacological target for antinociception. However, the role of PPAR-γ activation in the development of peripheral neuropathic pain is not fully understood.

Thus, in an effort to understand the contribution of PPAR-γ activation to the development of neuropathic pain, the present study was designed to evaluate the effect of pioglitazone, a PPAR-γ agonist, in chronic constriction nerve injured rats. Additionally, to gain a better insight into the mechanisms of action of pioglitazone, we also investigated its effects on oxidative stress and inflammation. In addition, to elucidate whether the protective effects of pioglitazone were related to activation of PPAR-γ using bisphenol A diglycidyl ether (BADGE), a PPAR-γ antagonist. Finally, long-lasting effects of pioglitazone were also evaluated in mononeuropathic rats.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Wistar rats (160-200 g) of either sex were housed under standard conditions of light and dark cycle in the Central Animal House of I.S.F College of Pharmacy, Moga, India with food and water *ad libitum*. Animals were acclimatized to laboratory conditions before the behavioral tests. All experiments were carried between 08:00 and 16:00 hr. Each animal was used for a single treatment and each group consisted of six to eight animals and they were placed in group of three in each polypropylene plastic cage after surgery with husk bedding.

2.2 Drugs and Chemicals

Pioglitazone hydrochloride (Dr. Reddy's Labs, India), BADGE (bisphenol A diglycidyl ether) (Sigma-Aldrich Corporation, India), and Evans blue (EB) were used in this study. Rat TNF-α

and IL-1β ELISA kits (R&D systems, MN, USA) were used to quantify cytokines. Unless stated, all other chemicals and biochemical reagents of highest analytical quality were used. Pioglitazone for intraperitoneal (i.p.) administration was freshly prepared by suspending in one or two drops of Tween 80 in normal saline. BADGE was dissolved in ethanol and diluted with 1:20 ethanol CMC (1.5% w/v in normal saline). The solutions were administered 0.5 mL per 100 g rat. Evans blue was dissolved in normal saline.

2.3 Chronic Constriction Injury (CCI) Model of Neuropathic Pain

The unilateral mononeuropathy was produced according to the method described by Bennett and Xie [17]. Briefly, the rats were anesthetized using thiopental sodium (30 mg/kg i.p.) and the common sciatic nerve of the left hind paw was exposed at the level of the middle of the thigh by blunt dissection through the biceps femoris muscle. Proximal to the sciatic trifurcation, approximately 7-mm of nerve was freed and 4 ligatures of 4-0 chromic gut were placed around the sciatic nerve with 1-mm intervals. Great care was taken not to interrupt epineural blood flow during tying the ligatures. In sham-operated rats, the same surgical procedure was followed, the connective tissue was freed, and no ligatures were applied. After surgery, all animals received gentamicin (5 mg/kg, i.p.) to prevent sepsis. Body weight and locomotor activity score were measured on day 0 before surgery, day 14 (in pre-emptive paradigm), and day 28 after surgery (in long lasting recovery groups).

2.4 Treatment Schedule

All animals were acclimatized to laboratory environment for at least 2 hr before behavioral testing. The experimental protocol consists of two treatment paradigms i.e., one was pre-emptive paradigm and the other was long lasting recovery paradigm. The pre-emptive paradigm comprised of seven groups, namely group I: sham control, group II: CCI control, group III: CCI + pioglitazone 3 mg/kg, group IV: CCI + pioglitazone 10 mg/kg, group V: CCI + pioglitazone 30 mg/kg, VI: CCI + BADGE 30 mg/kg, V: CCI + BADGE 30 mg/kg + pioglitazone 30 mg/kg. The long lasting recovery paradigm comprised of three groups, namely group I: sham control, group II: CCI control, group III: CCI + pioglitazone 30 mg/kg. All the rats were subjected to these two behavioral pain tests cold allodynia and thermal hyperalgesia on day 0

before performing surgery and subsequently 2 hr after vehicle or pioglitazone administration on the specified days. To evaluate the pre-emptive effects of pioglitazone (3, 10 or 30 mg/kg, i.p., once daily) on development of neuropathic pain symptoms in rats, treatment was initiated 1 hr before surgery and continued for two weeks. Further, in the antagonist studies, separate groups of CCI rats were administered BADGE alone (30 mg/kg, i.p.) or BADGE (30 mg/kg, i.p.) 30 min before pioglitazone (30 mg/kg, i.p.) administration. Sham-operated and nerve-injured control animals (CCI control) received equal volume of vehicle before the nociceptive stimulus at time when pioglitazone was administered. The response to behavioral nociceptive tests was assessed on days 0, 3, 7, 10 and 14 following surgery. Further, to study long-lasting effects of pioglitazone on the development of neuropathic pain, CCI rats were administered pioglitazone (30 mg/kg, i.p.) 1 hr before surgery and continued once daily for two weeks and without treatment for the next two weeks after surgery (recovery group). These animals were assessed for development of neuropathic pain at weekly intervals for 4 weeks following surgery.

2.5 Behavioral Test Paradigm

2.5.1 Assessment of cold allodynia

Cold allodynia was evaluated as the withdrawal latency (sec) to thermal, non-noxious stimulus of hind paws when dipped in water bath maintained at $10 \pm 0.5°C$ [18]. Baseline latency of paw withdrawal to cold stimulation was established thrice, 5 min apart, and averaged. A cut-off time of 15 sec was imposed. A significant reduction in paw withdrawal latency (PWL) indicates allodynia.

2.5.2 Assessment of thermal hyperalgesia

The mean PWL (sec) of the rat paw when dipped in water bath maintained at $47 \pm 0.5°C$ was measured. The baseline latency of paw withdrawal from thermal source was established three times, 5 min apart, and averaged. A cut-off time of 15 sec was imposed to avoid injury to the paw. The change in the PWLs as compared to basal responses was calculated as a measure of hyperalgesia in all the groups [19].

2.6 Plasma Extravasation

Evans blue dye (EB) intravenous (*i.v.*, in the caudal tail vein) injection (75 mg/kg *i.v.*) was used to measure plasma extravasation. On days 14 and 28 after surgery, rats were injected EB one hour after vehicle or pioglitazone treatment in pre-emptive paradigm and long-lasting recovery groups. After one hour, rats were sacrificed by cervical dislocation, and ipsilateral and contralateral paws excised. Plasma extravasion was determined as described by La Rana (2008) with slight modifications. Both hind paws (ipsi- and contralateral) were divided separately into sections and extracted with 4 mL of formamide for 72 hr and thereafter the absorbance was measured at 550 nm (UV-1 Spectrophotometer, Thermo Electron Corporation, England). These values were obtained using a standard curve in order to determine the amount of EB dye in each sample and are expressed as µg EB per g tissue [20].

2.7 Collection of Sciatic Nerve in Rats

The animals were sacrificed by cervical dislocation immediately after behavioral assays on day 14 (pre-treatment groups) and 28 (Long-lasting recovery group). In this study, at the end of treatment schedule in all the treatment groups, ipsilateral sciatic nerve of each rat was collected for the estimation of markers of nitroso-oxidative stress and inflammation. The isolated sciatic nerve was weighed, homogenized in ice cold phosphate buffer pH 7.0 and divided in to two portions. One part of the homogenate was centrifuged for 15 min at $2000g$ to obtain the clear supernatant for the estimation of nitroso-oxidative stress markers and another part of homogenate was mixed 4 µL/mL protease inhibitor cocktail. These samples were cold centrifuged at $14,000g$ at 4 °C for 15 min and the supernatant was used for estimation of pro-inflammatory cytokines.

2.8 Measurement of Pro-inflammatory Cytokines

The supernatant of sciatic nerve homogenate was used for the estimation of levels of IL-1β and TNF-α. Samples are estimated using the quantitative sandwich enzyme immunoassay according to manufacturer's instructions (R&D systems, MN, USA). The cytokine level was determined by comparing samples to the standard curve generated from the respective kits at 450 nm and are expressed as pg per mg wet weight of sciatic nerve.

2.9 Estimation of Markers of Oxidative Stress

(a) Lipid peroxidation in spinal cord was estimated colourimetrically by measuring thiobarbituric acid reactive substances (TBARS) by the method of Niehaus and Samuelson [21]. The values are expressed as nmol/g tissue. (b) Protein carbonyl content was determined by a method described by Levine [22]. The values are expressed as µmole/g tissue. (c) The concentration of endogenous antioxidant reduced glutathione (GSH) level was estimated following the method described by Lou et al. [23]. The values are expressed as µmole/g tissue. Activities of (d) catalase and (e) superoxide dismutase (SOD were measured by the methods of Aebi [24] and Misra and Fridovich [25], respectively and expressed as % activity of sham control. (f) The nitrite concentration was measured by the Griess reaction [26] and are expressed in nmole/g tissue.

2.10 Statistical Analysis

The results are expressed as mean ± SEM of n (number of animals studied) observations. The data were analyzed using one-way ANOVA followed by Tukey test for multiple comparisons. $P < 0.05$ was considered significant.

3. RESULTS

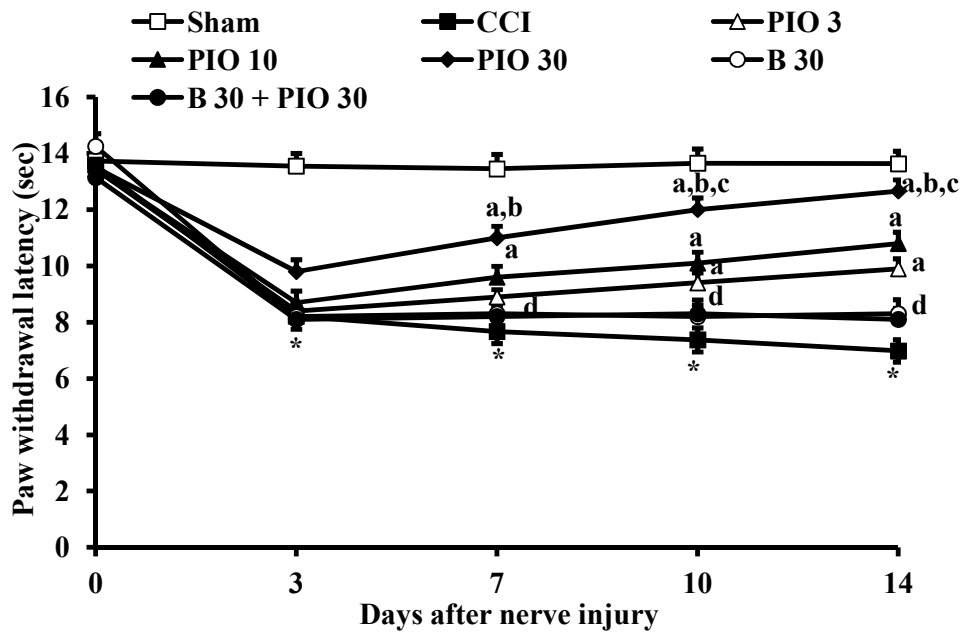### 3.1 Chronic Constriction Injury and Induction of Neuropathy

The baseline paw withdrawal response in each test obtained on day 0 for each rat was relatively stable and showed no significant variation. Following surgery, the rats kept their nerve injured paw elevated above the cage floor, but otherwise appeared healthy, exhibited normal grooming and feeding behavior, and gained weight normally. The ipsilateral and contralateral paw withdrawal responses to thermal and cold stimulation in sham-operated rats remained unchanged from baseline values throughout the entire observation period. The contralateral PWLs in response to thermal or cold stimulation did not alter in CCI control rats as compared to that of sham-operated rats (Data not shown).The ipsilateral paw withdrawal responses of all the CCI rats were significantly less than that of sham-operated rats on day 3 onwards and reached steady state between days 14 and 28 after surgery indicating the development and of allodynia and hyperalgesia in a time-dependent manner (Figs. 1 and 2).

3.2 Effect of Pioglitazone on Motor Activity

CCI rats did not show any effect on locomotor activity as compared to sham-operated rats. Administration of pioglitazone (3, 10 or 30 mg/kg, i.p., for 2 weeks) had no effect on motor activity in the CCI rats as compared to CCI control rats. BADGE alone (30 mg/kg, i.p.) or prior to pioglitazone (30 mg/kg, i.p.) did not alter locomotor activity in CCI rats (data not shown). Further, animals treated with pioglitazone (30 mg/kg, i.p.) had shown normal motor activity during the recovery period (data not shown).

3.3 Effect of Pioglitazone on Cold Allodynia and Thermal Hyperalgesia in CCI Rats

Before chronic constriction injury, all groups exhibited comparable base line latencies to cold and hot thermal stimuli. Sham surgery did not produce any significant behavioral hypersensitivity. CCI rats showed significantly reduced latencies to ipsilateral paw withdrawal upon application of cold and hot thermal stimuli indicating the development of neuropathic pain state, allodynia (Fig. 1) and hyperalgesia (Fig. 2), in comparison to sham-operated rats. Pre-emptive pioglitazone (3, 10 or 30 mg/kg) dose-dependently attenuated the development of pain hypersensitivities in CCI rats (Figs. 1 and 2). BADGE alone (30 mg/kg, i.p.) did not alter PWLs to both the stimuli in CCI rats. However, the antiallodynic and antihyperalgesic effects of pioglitazone (30 mg/kg) were abolished by pre-administration of BADGE (30 mg/kg) in CCI rats on day 14 (Figs. 1 and 2). In all these treatment groups, pioglitazone with or without BADGE had no effect on the contralateral PWLs in these behavioral tests as compared to that of CCI rats (data not shown). Furthermore, to assess whether the discontinuation of pioglitazone administration results in return of the behavioral symptoms of neuropathic pain or in maintenance of antiallodynic and antihyperalgesic effects, rats were subjected to behavioral assays of neuropathic pain during the next two weeks following the treatment termination (recovery). The relief of allodynia (Fig. 3) and hyperalgesia (Fig. 4) elicited by the highest dose of pioglitazone (30 mg/kg) did not disappear even after the discontinuation of the treatment throughout the remaining observation time.
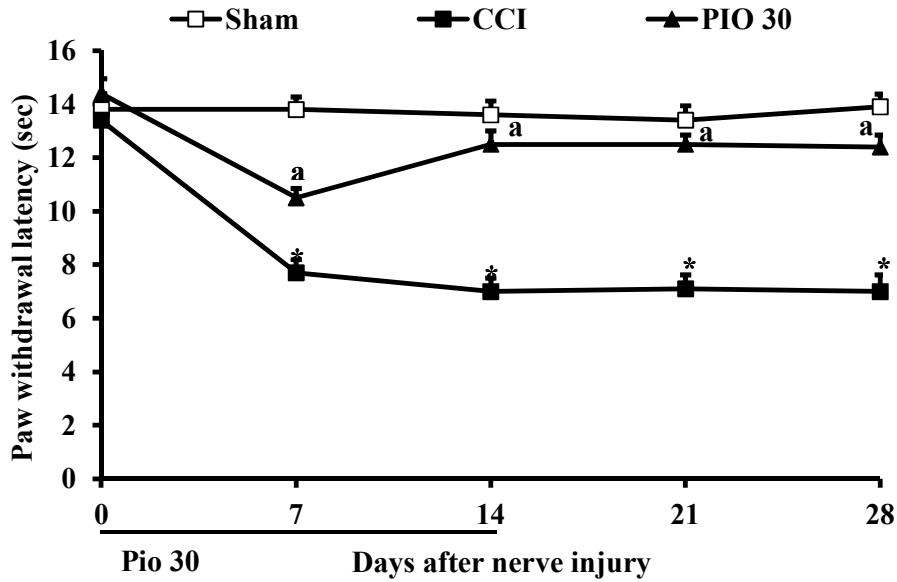
Fig. 1. Effect of pre-emptive pioglitazone on ipsilateral paw withdrawal latencies to cold stimuli in chronic constriction injury (CCI) rats

PIO: Pioglitazone (3, 10 or 30 mg/kg); B 30: BADGE 30 mg/kg. Values are mean ± SEM. [*]P < 0.05 vs Sham control; [a]P < 0.05 vs CCI control; [b]P < 0.05 vs PIO 3; [c]P < 0.05 vs PIO 10; [d]P < 0.05 vs PIO 30
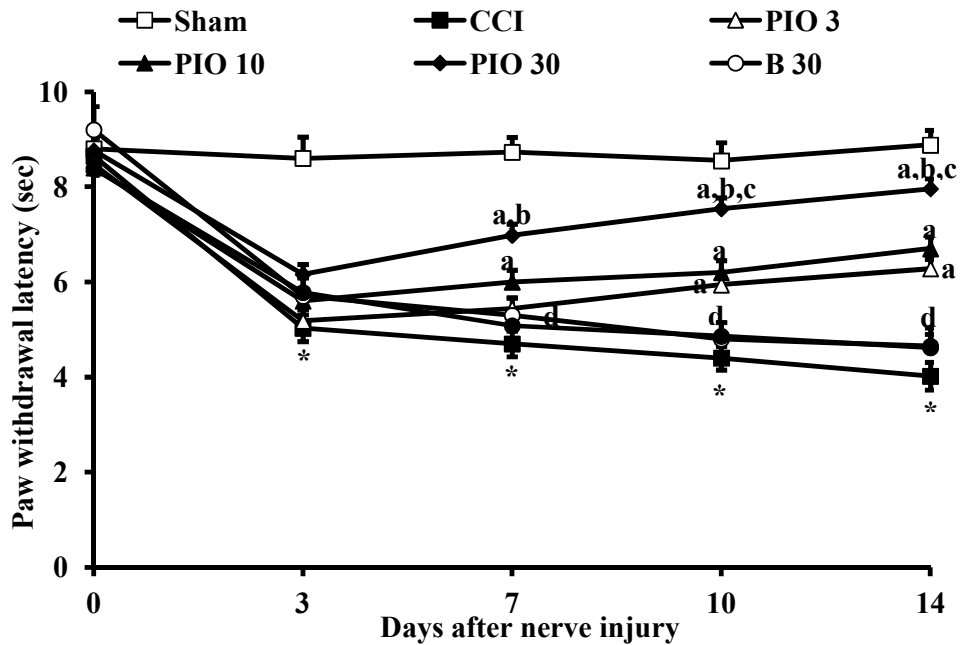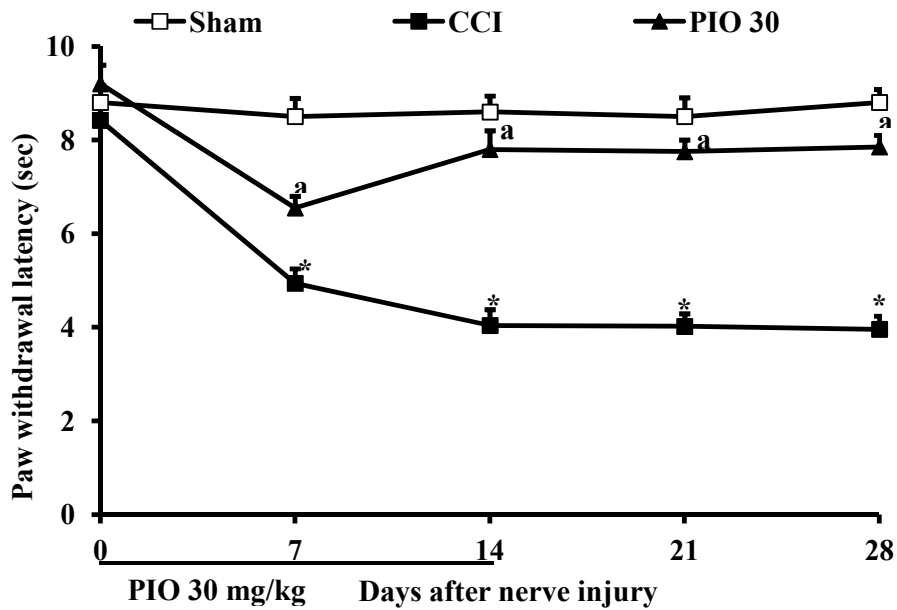

Fig. 2. Effect of pre-emptive pioglitazone on ipsilateral paw withdrawal latencies to thermal stimuli in chronic constriction injury (CCI) rats

PIO: Pioglitazone (3, 10 or 30 mg/kg); B 30: BADGE 30 mg/kg. Values are mean ± SEM. [*]P < 0.05 vs Sham control; [a]P < 0.05 vs CCI control; [b]P < 0.05 vs PIO 3; [c]P < 0.05 vs PIO 10; [d]P < 0.05 vs PIO 30

Fig. 3. Effect of pioglitazone on ipsilateral paw withdrawal latencies to cold stimuli in chronic constriction injury (CCI) rats (recovery)
PIO 30: Pioglitazone 30 mg/kg. Values are mean ± SEM. $^*P < 0.05$ vs Sham control; $^aP < 0.05$ vs CCI control

Fig. 4. Effect of pioglitazone on ipsilateral paw withdrawal latencies to thermal stimuli in chronic constriction injury (CCI) rats (recovery)
PIO 30: Pioglitazone 30 mg/kg. Values are mean ± SEM. $^*P < 0.05$ vs Sham control; $^aP < 0.05$ vs CCI control

3.4 Effect of Pioglitazone on Plasma Extravasation in CCI Rats

CCI rats showed increased vascular permeability as measured by increased Evans blue extravasation in ipsilateral paw as compared to that of sham-operated rats. However, there was no significant difference in capillary permeability between contralateral paws of sham-operated and CCI rats. Pre-emptive pioglitazone (3, 10 or

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30 mg/kg) significantly and dose-dependently reduced the plasma extravasation in ipsilateral paw of CCI rats in comparison to that of CCI control rats on day 14 (Fig. 5). BADGE alone (30 mg/kg, i.p.) did not alter capillary permeability in CCI rats. However, treatment of CCI rats with BADGE (30 mg/kg) prior to pioglitazone (30 mg/kg) significantly prevented the effect of pioglitazone (Fig. 5). Conversely, pioglitazone at any dose had no effect on plasma extravasation in contralateral paw of CCI rats as compared to that of CCI control rats (Fig. 5). Further, in recovery group, pioglitazone (30 mg/kg) significantly reduced the plasma extravasation in ipsilateral paws, but not in contralateral paws of CCI rats as compared to that of CCI control rats (Fig. 6).

3.5 Effect of Pioglitazone on Pro-inflammatory Cytokines

The levels of TNF-α and IL-1β were significantly elevated in ipsilateral sciatic nerve of CCI rats as compared to that of sham-operated rats. Following pre-emptive administration of pioglitazone (3, 10 or 30 mg/kg) dose-dependently and significantly reduced the increased levels of IL-1β and TNF-α in ipsilateral sciatic nerve of CCI rats as compared to that of CCI control rats on day 14. Though BADGE alone (30 mg/kg, i.p.) had no effect, but pretreatment of rats with BADGE (30 mg/kg) diminished the effects of pioglitazone (30 mg/kg)

on TNF-α and IL-1β levels in the injured sciatic nerve of CCI rats (Table 1). In recovery groups, pioglitazone (30 mg/kg) significantly reduced the levels of TNF-α and IL-1β in injured sciatic nerve of CCI rats as compared to that of CCI control rats (Table 1).

### 3.6 Effect of Pioglitazone on the Markers of Oxidative and Nitrosative Stress

Sciatic nerve injury state led to increased levels of nitroso-oxidative stress markers accompanied by marked reduction in antioxidant defenses injured nerve in comparison to sham-surgery (Tables 2 and 3). Pre-emptive pioglitazone (3, 10 or 30 mg/kg) dose-dependently and significantly reduced elevated levels of TBARS, protein carbonyl, and nitrite (Table 2), restored the depleted level of GSH, and improved SOD and catalase activities (Table 3) in the injured nerve of CCI rats in comparison to that of CCI control rats on day 14. BADGE alone was without any effect on these nitroso-oxidative stress markers, whereas pretreatment with BADGE (30 mg/kg, i.p.) significantly reduced the effect of pioglitazone (30 mg/kg) on nitroso-oxidative stress markers (Tables 2 and 3). In recovery groups, pioglitazone (30 mg/kg) significantly reduced TBARS, protein carbonyl, and nitrite levels and improved the level of GSH and activities of SOD and catalase in injured nerve of CCI rats as compared to that of CCI control rats (Table 4).

Table 1. Effect of pioglitazone on tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in chronic constriction injury (CCI) rats

Group	TNF-α (pg/mg weight)	IL-1β (pg/mg wet weight)
Pre-emptive paradigm		
Sham control	85.6 ± 8.2	105.8 ± 11.5
CCI control	379.8 ± 25.8[*]	485.7 ± 31.9[*]
PIO 3	280.1 ± 19.1[a]	375.6 ± 27.6[a]
PIO 10	189.3 ± 16.4[a,b]	249.7 ± 20.7[a,b]
PIO 30	121.4 ± 17.3[a,b,c]	146.6 ± 23.9[a,b,c]
B 30	391.2 ± 14.8	456.5 ± 23.9
B 30 + PIO 30	349.7 ± 22.1[d]	461.7 ± 20.4[d]
Long lasting recovery		
Sham control	77.8 ± 11.1	95.8 ± 9.9
CCI control	360.3 ± 28.3[*]	425.2 ± 25.9[*]
PIO 30	158.5 ± 16.5[a]	191.5 ± 20.5[a]

PIO: Pioglitazone (3, 10 or 30 mg/kg); B 30: BADGE 30 mg/kg. Values are mean ± SEM. [*] $P < 0.05$ vs Sham control; [a] $P < 0.05$ vs CCI control; [b] $P < 0.05$ vs PIO 3; [c] $P < 0.05$ vs PIO 10; [d] $P < 0.05$ vs PIO 30
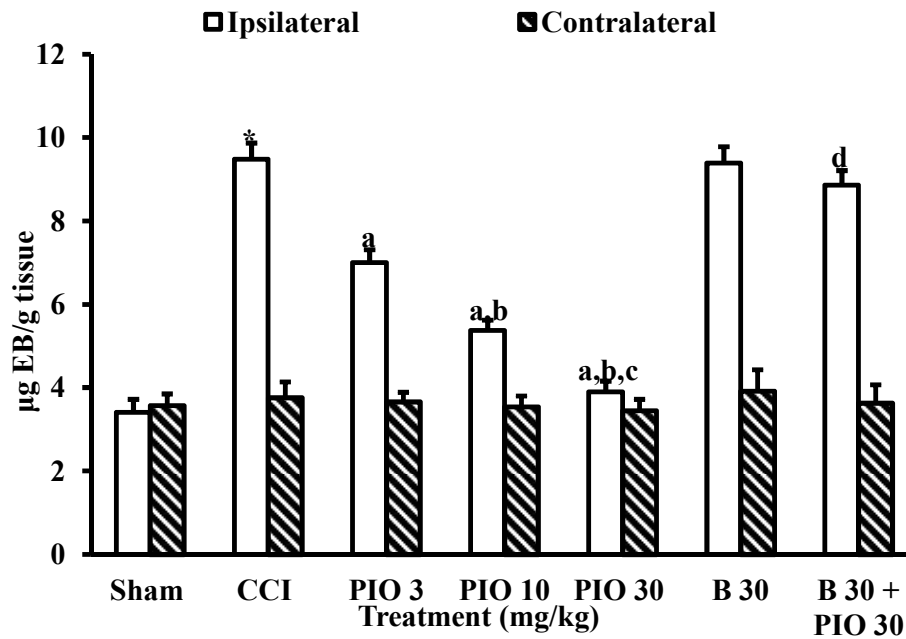
Fig. 5. Effect of pre-emptive pioglitazone on plasma extravasation in chronic constriction injury (CCI) rats

*PIO: Pioglitazone (3, 10 or 30 mg/kg); B 30: BADGE 30 mg/kg. Values are mean ± SEM. *P < 0.05 vs Sham control; aP < 0.05 vs CCI control; bP < 0.05 vs PIO 3; cP < 0.05 vs PIO 10; dP < 0.05 vs PIO 30*
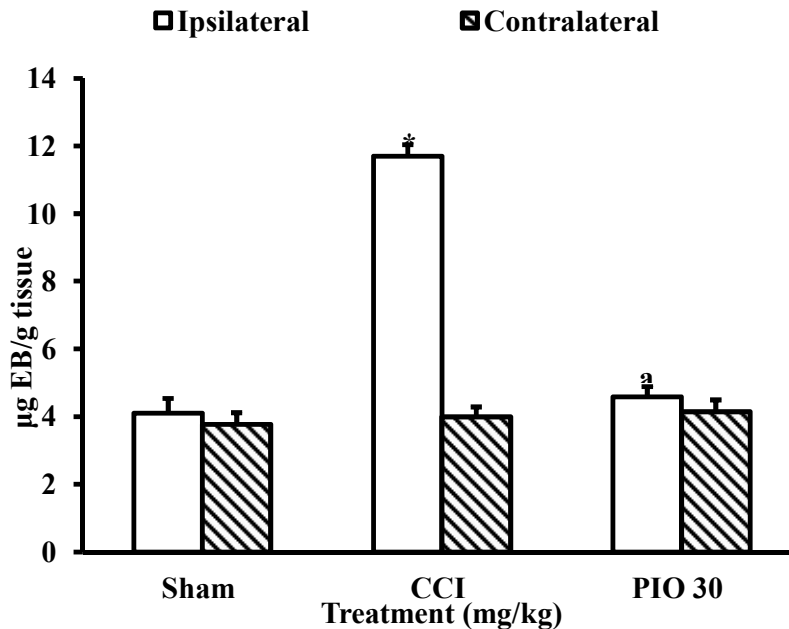
Fig. 6. Effect of pioglitazone on plasma extravasation in chronic constriction injury (CCI) rats (recovery)

*PIO 30: Pioglitazone 30 mg/kg. Values are mean ± SEM. *P < 0.05 vs Sham control; aP < 0.05 vs CCI control*

Table 2. Effect of pre-emptive pioglitazone on TBARS, protein carbonylation and nitrite levels in chronic constriction injury (CCI) rats

Treatment (mg/kg)	TBARS (nmole/g tissue)	Protein carbonylation (μmole/g tissue)	Nitrite (nmole/g tissue)
Sham control	75.40 ± 15.73	1.12 ± 0.21	83.84 ± 16.69
CCI control	315.86 ± 22.41[*]	3.98 ± 0.16[*]	315.41 ± 19.73[*]
PIO 3	247.86 ± 10.86[a]	2.78 ± 0.17 [a]	225.75 ± 12.97[a]
PIO 10	179.05 ± 10.36[a,b]	1.98 ± 0.21 [a,b]	163.16 ± 5.24[a,b]
PIO 30	93.64 ± 12.33[a,b,c]	1.10 ± 0.17 [a,b,c]	92.82 ± 6.49[a,b,c]
B 30	293.70 ± 19.81	3.54 ± 0.18	296.90 ± 21.62
B 30 + PIO 30	272.76 ± 10.87[d]	2.98 ± 0.13 [d]	274.20 ± 10.58[d]

TBARS: Thiobarbituric acid reacting substances; PIO: Pioglitazone (3, 10 or 30 mg/kg); B 30: BADGE 30 mg/kg. Values are mean ± SEM. [*]P < 0.05 vs Sham control; [a]P < 0.05 vs CCI control; [b]P < 0.05 vs PIO 3; [c]P < 0.05 vs PIO 10; [d]P < 0.05 vs PIO 30.

Table 3. Effect of pre-emptive pioglitazone on GSH, SOD, and catalase in chronic constriction injury (CCI) rats

Treatment (mg/kg)	GSH (μmole/g tissue)	SOD (% Sham control)	Catalase (% Sham control)
Sham control	5.01 ± 0.29	100.04 ± 7.43	99.98 ± 9.81
CCI control	2.25 ± 0.15[*]	40.17 ± 3.69*	36.64 ± 2.26[*]
PIO 3	3.30 ± 0.17 [a]	61.43 ± 4.41 [a]	60.68 ± 3.52 [a]
PIO 10	4.00 ± 0.19 [a]	70.28 ± 3.15 [a]	69.52 ± 4.14 [a]
PIO 30	4.98 ± 0.28 [a,b,c]	92.25 ± 3.85 [a,b,c]	92.93 ± 4.72 [a,b,c]
B 30	2.23 ± 0.19	45.10± 2.37	44.66 ± 3.10
B 30 + PIO 30	2.64 ± 0.19 [d]	51.73± 3.94 [d]	54.88 ± 3.63 [d]

GSH: Reduced glutathione; SOD: Superoxide dismutase; PIO: Pioglitazone (3, 10 or 30 mg/kg); B 30: BADGE 30 mg/kg. Values are mean ± SEM. [*]P < 0.05 vs Sham control; [a]P < 0.05 vs CCI control; [b]P < 0.05 vs PIO 3; [c]P < 0.05 vs PIO 10; [d]P < 0.05 vs PIO 30

Table 4. Effect of pioglitazone on markers of oxidative and nitrosative stress in chronic constriction injury (CCI) rats (recovery)

Marker	Sham control	CCI control	PIO 30
TBARS (nmole/g tissue)	87.60 ± 10.96	265.85 ± 18.35[*]	121.73 ± 13.67[a]
Protein carbonylation (μmole/g tissue)	0.66 ± 0.12	2.65 ± 0.22[*]	0.97 ± 0.18[a]
Nitrite (nmole/g tissue)	83.71 ± 11.46	381.51 ± 31.15[*]	157.18 ± 16.23[a]
GSH (μmole/g tissue)	5.28 ± 0.75	1.96 ± 0.23[*]	4.01 ± 0.65 [a]
SOD (% Sham control)	100.15 ± 9.54	45.95 ± 4.75[*]	81.60 ± 7.49 [a]
Catalase (% Sham control)	99.83 ± 8.76	38.14 ± 2.91[*]	84.97 ± 7.16 [a]

TBARS: Thiobarbituric acid reacting substances; GSH: Reduced glutathione; SOD: Superoxide dismutase; PIO: Pioglitazone (3, 10 or 30 mg/kg). Values are mean ± SEM. [*]P < 0.05 vs Sham control; [a]P < 0.05 vs CCI control

4. DISCUSSION

In the resent study, pre-emptive administration of pioglitazone, a PPAR-γ agonist was effective in blocking the development of cold allodynia and thermal hyperalgesia following nerve injury. It is important to note that pre-emptive treatment with pioglitazone during the development of neuropathic pain did not alter body weight and locomotor activity during the study period indicating that the observed effects of pioglitazone were not due to any effect on

neurobehavioral and general growth changes. In addition, pioglitazone did not affect behavioral response to thermal and cold stimuli in the contralateral paw of mononeuropathic rats signifying that the doses employed were not directly antinociceptive and the observed effects in the ipsilateral paw of mononeuropathic rats were not due to hypoalgesic effects as well. It is further supported by the previous studies that pioglitazone did not show any effect in hot plate, tail flick tests and the first phase of the formalin test that involve central sensory processing [27].

In one study, it was reported that spinal administration of PPAR-γ agonist rosiglitazone decreased the paw withdrawal latencies to cold and mechanical stimuli in spare nerve injury model whereas it did not alter such response in naïve animals [28]. In the same study, it has been reported that only spinal administration of rosiglitazone (100 μg/animal) had shown ameliorative effect on neuropathic pain whereas no effect was observed following intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) administration. One limitation of this study was that the same dose was used in all the three routes of administration. Although rosiglitazone is more potent PPAR-γ agonist in that it binds to the receptor with ten times more affinity (K_d of ~40nM) than pioglitazone (K_d of ~400nM) [29], however, pioglitazone cross the blood-brain barrier much more easily than rosiglitazone [30], which may indicate that higher doses of rosiglitazone might be needed to achieve the same degree of protection as that induced by pioglitazone. When given peripherally, moderate levels of pioglitazone (~18%) enter the CNS [30] and peripherally administered pioglitazone has also shown protection in various CNS diseases [14,15,31]. It is possible that both peripheral and central mediated mechanisms involved in antinociceptive effects of pioglitazone. These data along with results of the present study clearly demonstrate that systemically administered pioglitazone alleviated the development of neuropathic pain. Further, pretreatment of animals with BADGE, a PPAR-γ antagonist attenuated the protective effects of pioglitazone in mononeuropathic rats. Collectively, the results reported here confirm that nuclear PPAR-γ activation contributes to the antihyperalgesic and antiallodynic effects and further attenuates the development of neuropathic pain.

Of particular note, complex mechanisms and mediators of oxidative and nitrosative stress are implicated in the development and maintenance of neuropathic pain. Nerve injury leads to the recruitment of immune and inflammatory cells such as mast cells, macrophages and neutrophils at the site of injury which increase the release of free radicals [1,3]. Accumulating data indicate that peripheral nerve injury causes upregulation in both the expression and activity of inducible nitric oxide synthase (NOS) and neuronal NOS in sciatic nerve and dorsal root ganglia and also increased the levels of ROS and nitrite and diminished antioxidant defenses in sciatic nerve and injured paw in mononeuropathic rats [5,7,32]. Moreover, SOD converts superoxide anion radicals produced in the body to hydrogen peroxide, thereby reducing the likelihood of superoxide anion interacting with NO to form reactive peroxynitrite. Indeed, peroxynitrite directly causes the hypersensitization and nitrosative stress [33]. Consistent to previous studies, there was a marked oxidative stress after nerve injury and pre-emptive administration of pioglitazone reduced oxidative stress, decreased the level of nitrite, and restored antioxidant defenses by increasing GSH and improving activities of SOD and catalase in injured sciatic nerve. Importantly, such antioxidant effects of pioglitazone were sustained even after termination of treatment for the remaining study period.

There is a strong correlation between oxidative stress and the development of neuropathic pain following nerve injury. Indeed, free radicals and end products of lipid and protein oxidation are also known to cause neuronal hypersensitivity following tissue injury [6,34,35]. Moreover, activation of PPAR-γ provides neuroprotection by inhibiting free radical generation and improving the antioxidant defense factors such as GSH, SOD, catalase, and glutathione peroxidase under various pathological conditions associated with inflammation [15,16,31,36]. It has been reported that catalase and SOD gene promoters contain the PPRE indicating that they are directly regulated by PPAR-γ. Further, there is now evidence indicating that PPAR-γ agonists exert direct and rapid effects on mitochondrial respiration by inhibiting complex I and complex III activities [35]. Recent evidence suggests that nuclear factor-erythroid 2-related factor/antioxidant responsive element (NRF2/ARE) and PPAR-γ/PPAR-γ response element (PRE) pathways regulate redox signaling and the underlying mechanisms to control oxidative stress, inflammation and overcome mitochondrial impairment [37,38].

Indeed, synthetic and natural agents that abrogate nitroso-oxidative stress and free radical scavengers were well reported to inhibit neuropathic pain [34,38-41]. It is possible that pioglitazone activated antioxidant mechanisms might be contributed to attenuate the development of neuropathic pain and sustained long-term effects and such protective effects were abolished by PAPR-γ antagonist BADGE in ipsilateral paws of mononeuropathic rats. Thus, previous findings along with the results of the present study support the evidence that PPAR-γ activation exerted long-lasting effects and prevented the development of neuropathic pain following nerve injury, at least, in part by inhibiting nitroso-oxidative stress.

Neuropathic pain consequent to peripheral nerve injury has also been associated with local inflammation. It is well reported that noxious stimulation of afferent fibers releases substance P, calcitonin-gene related peptide, PGs, and NO which causes increased vascular permeability that leads to leakage of plasma fluid [20]. Indeed, CCI-induced plasma extravasation at early days after ligation is caused by the release of neuroactive peptides at peripheral levels, whereas other inflammatory mediators such as PGs, NO and pro-inflammatory cytokines are involved subsequently [18,20,39,42]. The present results reveal that pioglitazone dose- and receptor-dependently inhibited plasma extravasation in the ipsilateral paw of CCI animals as pretreatment with BADGE attenuated the anti-inflammatory effect of pioglitazone. The exact mechanisms involved in this effect are not clear. One possible explanation is that there is strong recruitment of inflammatory cells such as mast cell, macrophages and neutrophils at the site of nerve injury which participate to the following inflammation evoked by CCI of the sciatic nerve [1,3]. Moreover, PPAR-γ are expressed on mast cell, macrophages and neutrophils and activation of PPAR-γ inhibits inflammatory cell infiltration [42]. Therefore, activation of PPAR-γ results in decreased plasma protein leakage that leads to inhibition of sensitizing substances release thereby reduces inflammation following nerve injury.

Growing body of data indicate that pro-inflammatory cytokines play an important role in the development of hypersensitivity after nerve injury and inflammation [2,43]. Numerous studies emphasized on the importance of neuroimmune interactions in neuropathic pain as evidenced by the involvement of different immune cells in peripheral and central sensitization of pathological pain [2,39,43,44]. Upon nerve injury, non-neuronal cells, both astrocytes and microglia are activated in the spinal cord. Both TNF-α and IL-1β have been shown to evoke pain and their inhibitors have been found to reduce pain hypersensitivity in neuropathic pain [2,4,44,45]. In line with these studies, there was a marked inflammation observed in the injured sciatic nerves and pre-emptive pioglitazone administration markedly reduced TNF-α and IL-1β levels and such inhibitory effects were sustained until the end of the observation period. These effects were abolished by BADGE indicating PPAR-γ receptor-dependent anti-inflammatory effects.

It is also observed that pioglitazone exhibited long-lasting anti-inflammatory effects that parallels with the attenuation of the development of neuropathic pain hypersensitivity. It is well reported that nerve injury leads to the activation of macrophages, microglia and Schwann cells that enhance the release pro-inflammatory cytokines [2,4,44,46]. Activated glial cells releases pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6, which is the beginning of a complex neuroinflammatory cascade that enhances and maintains the neuropathic pain [44-46]. A rapidly growing data stream indicates that PPAR-γ activation inhibits the activation of macrophage, microglia and neurons and thus prevents the induction of NF-κB and inhibits the expression of various inflammatory proteins such as iNOS, COX-2, pro-inflammatory cytokines TNF-α and IL-1β [11-13,40]. Also, PPAR-γ agonists alleviated chronic inflammation as well as various neurodegenerative diseases associated with inflammation in both the periphery and the CNS [13-16,31,47]. It is therefore plausible that activation of PPAR-γ associated anti-inflammatory mechanisms are sustained and contributed to attenuation of development of neuropathic behavioral hypersensitivity.

5. CONCLUSION

The evidence arising from all the findings presented herein, pre-emptive administration of pioglitazone abolished the development of neuropathic pain and showed sustained long-lasting anti-nociceptive effect after termination of pioglitazone administration. Further, these preventive and long-lasting effects were associated with reduction in peripheral inflammation and pro-inflammatory mediators as

well as nitroso-oxidative stress. Adding to this, PPAR-γ antagonist BADGE abolished these protective effects of pioglitazone. Thus, the results confirm that a key role of pre-emptive PPAR-γ activation in abolishing or at least delaying the development of neuropathic pain after nerve injury.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experimental protocols were approved by the Institutional Animal Ethics Committee (CPPU/0508/PTMP-PCS/0608/1) and were carried out in accordance with the guidelines of the Indian National Science Academy for the use and care of experimental animals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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