



Nuclear PPAR- γ Activation Modulates Inflammation and Oxidative Stress in Attenuating Chemotherapy-Induced Neuropathic Pain *in Vivo*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors HP and PVSRGK conceptualized the study. Authors HP, SSV and PVSRGK performed the methodology. Author HP did the investigation and data analysis of the study. Authors HP and PVSRGK did data validation of the study. Author HP wrote the original draft of the manuscript. Authors HP, SSV, SD and PVSRGK wrote, reviewed and edited the manuscript. All authors take responsibility for appropriate content, critically revised the manuscript, and approved the version of the manuscript to be published.

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ABSTRACT

Background: Paclitaxel-induced painful neuropathy is a major dose-limiting side effect and can persist for up to two years after completing treatment that greatly affects both the course of chemotherapy and quality of life in cancer patients. Peroxisome proliferator-activated receptor (PPAR)- γ belongs to a family of nuclear receptors known for their transcriptional and regulatory roles in metabolism, inflammation, and oxidative stress. However, the role of PPAR- γ activation on paclitaxel-induced neuropathic pain is not yet known.

Objective: To investigate whether pioglitazone, a PPAR- γ agonist reduce paclitaxel-induced neuropathic pain and to elucidate underlying mechanisms.

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Methodology: Peripheral neuropathy was induced by administration of paclitaxel (2 mg/kg per injection) intraperitoneally on four alternate days (days 0, 2, 4, 6). Thermal hyperalgesia and mechanical allodynia were assessed and the markers of inflammation and nitroso-oxidative stress were estimated.

Results: Pioglitazone did not induce hypoalgesia and had no effect on locomotor activity. Repeated oral administration of pioglitazone (10 and 20 mg/kg,) for 2 weeks started 14 days after paclitaxel injection markedly attenuated paw withdrawal responses to thermal (hyperalgesia) and mechanical (allodynia) stimuli. Further, pioglitazone administration significantly reduced elevated level of pro-inflammatory cytokine, TNF- α , in both the dorsal root ganglia and the spinal cord accompanied by marked decrease in oxidative stress parameters as well as increase in activity of antioxidant defense enzyme, superoxide dismutase, in the spinal cord after paclitaxel injection.

Conclusion: The results of the present study demonstrate that pioglitazone, a PPAR- γ agonist exerted antinociceptive effect in paclitaxel-induced neuropathic pain through inhibiting neuroimmune inflammation in both the periphery and spinal cord and by reducing nitroso-oxidative stress in spinal cord. Our findings strongly suggest pharmacological activation of PPAR- γ as a promising therapeutic target in paclitaxel-induced peripheral neuropathy and provide rationale for the clinical evaluation.

Keywords: CIPN, Chemotherapy-induced; Paclitaxel; Paclitaxel neuropathy; Peripheral neuropathy; Pioglitazone; PPAR- γ agonist; Neuropathic pain.

1. INTRODUCTION

Chemotherapy-induced peripheral neuropathy (CIPN) is the most common side effect of typical cytotoxic anti-cancer agents, such as taxanes (paclitaxel, docetaxel), platinum compounds (cisplatin, oxaliplatin), and vinca alkaloids (vincristine, vinblastine). The rate of prevalence of CIPN is anticancer agent-dependent varying from 19% to more than 85% and that of taxanes-associated neuropathic pain ranges from 11 to 87% whereas 60-70% of paclitaxel received patients reported neuropathy as a side effect [1,2]. Paclitaxel, a secondary metabolite produced by *Taxus* sp., inhibits microtubule assembly, stabilizes the microtubule polymer, protects it from disassembly, thereby defects mitotic spindle assembly, chromosome segregation, and cell division [1,3]. Since its approval in 1998, paclitaxel has become one of the most commonly used chemotherapeutic agents for treating different types of cancers, especially breast, ovarian, lung, Kaposi's sarcoma, head, and leucopenia cancers [3]. Furthermore, it is often used as a reference to evaluate the therapeutic benefits achieved with the co-administration of another anticancer agent [2,3]. Notwithstanding to its broad chemotherapeutic spectrum, peripheral neuropathy, which typically presents as chronic debilitating pain, is a common adverse effect in paclitaxel use [1-3].

The pathophysiology of paclitaxel-induced neuropathic pain is not fully understood.

Essentially, paclitaxel-induced neuropathic pain is associated with the development of spontaneous activity and hyperexcitability in dorsal root ganglion (DRG) neurons that is paralleled by increased expression and function of voltage-gated sodium channels (Nav1.7) and low-voltage-activated calcium channels (T-type; Cav3.2) in DRG neurons [4]. It has been proposed that paclitaxel induce neuropathic pain, characterized by hyperalgesia and allodynia, due to its associated neurotoxic effects on intraepidermal nerve fibers, peripheral nerve damage, mitochondrial abnormality in sensory primary afferent neurons in DRG, and local perineuronal inflammation [5,6]. It has been reported that macrophage infiltration and neuroinflammation around DRG and release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-1 β leading to increased pain sensitivities following paclitaxel administration [7]. Growing body of evidence indicated involvement of activated non-neuronal cells, particularly microglia and astrocytes of the spinal cord in paclitaxel-induced peripheral neuropathy [8,9]. Several studies demonstrated role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the peripheral and central sensitization of neuropathic pain [10]. Essentially, CIPN symptoms persist for up to two years after discontinuation of treatment that significantly reduces patients' quality of life during the cancer treatment and in cancer survivals [2,3]. Nonetheless, the presently available therapeutic agents are partially effective and such therapies

are associated with unwanted effects that often limit their use. Therefore, there is an imperative need for the development of therapeutic agents capable of attenuating abnormal pain sensation without impairing anticancer effect of paclitaxel and also to improve the quality of life of cancer patients.

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and unique nuclear receptors that play key roles in the regulation of immune and inflammatory response [11]. Upon ligand binding the co-repressors dissociate from the PPAR/RXR complex, allowing for the recruitment of co-activators, 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 [11,12]. 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 [12]. Several studies identified that PPAR- γ are expressed on neutrophils, monocytes/macrophages, microglia, and neurons [11,12]. Moreover, 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)- κ B-dependent inflammatory genes [11]. Several PPAR- γ agonists, widely known as insulin sensitizers owing to their therapeutic effects, have been approved for the treatment of type-2 diabetes. Recent studies have shown PPAR- γ agonists ameliorate acute and chronic nociception and exert protection against inflammatory diseases of various etiologies include multiple sclerosis, Alzheimer's disease, Parkinson's disease, and spinal cord injury [12-14]. It has been reported that PPAR- γ activation alleviates oxidative stress and improves antioxidant defenses in diabetic neuropathy [15]. These data propose that PPAR- γ is a novel pharmacological target for modulating chronic nociceptive and inflammatory insults.

In the present study, we investigated whether pioglitazone, a PPAR- γ agonist attenuates neuropathic pain evoked by paclitaxel treatment. Secondly, we studied the mechanisms involved in the paclitaxel-induced neuropathic pain and further evaluated if the pharmacological PPAR- γ -

activation modulates such pathological mechanisms that may provide novel therapeutic modality in CIPN management.

2. MATERIALS AND METHODS

2.1 Animals

Male Wistar rats (180-200 g) were used in the present study. The animals were housed in groups of three, in polypropylene cages with husk bedding under standard conditions of light and dark cycle with standard laboratory rodent chow and water ad libitum. Animals were acclimatized to laboratory conditions before the test. All the behavioral assessments were carried between 9:00 AM and 11:00 AM. Each animal was used for a single treatment and each group consisted of six animals. All experiments for a given treatment were performed using age-matched animals in an attempt to avoid variability between experimental groups.

2.2 Drugs and Chemicals

Paclitaxel (Intaxel 100 mg Injection; Fresenius Kabi India Pvt. Ltd.) was freshly prepared by diluting in 0.9% saline to a concentration of 2 mg/ml prior to injection. Pioglitazone hydrochloride (Dr. Reddy's Labs, India) suspended in 0.5% carboxy methyl cellulose was orally administered through gastric lavage. Rat TNF- α ELISA kit (R&D systems, MN, USA) was used to quantify cytokine. Unless stated, all other chemicals and biochemical reagents of highest analytical grade quality were used. Treatment with either vehicle or pioglitazone 10 mg/kg and 20 mg/kg, per orally (p.o.) was initiated on day 14 after paclitaxel administration and was continued once daily for next 14 days.

2.3 Induction and Assessment of Paclitaxel-induced Peripheral Neuropathy in Rats

Rats were intraperitoneally (i.p.) injected with paclitaxel (2 mg/kg i.p. for 4 consecutive days i.e., on day 0, 2, 4, 6) to induce neuropathic pain [6]. Age-matched control rats received, in parallel, an equal volume of normal saline. The induction of neuropathic pain was confirmed by measurement of thermal hyperalgesia and mechanical allodynia. Body weights were measured using calibrated scale on day 0 (initial) and day 28 (final) and motor activity was systematically measured using actophotometer on days 0, 7, 14, 21 and 28 of the experiment.

2.4 Study Design

All animals were acclimatized to laboratory environment for at least 30 min on two or three separate days before testing. The experimental protocol comprised of five groups, namely group I: control (age-matched), group II: paclitaxel treated, group III: pioglitazone *per se* (20 mg/kg), group IV: paclitaxel + pioglitazone 10 mg/kg, and group V: paclitaxel + pioglitazone 20 mg/kg. Following habituation and baseline testing, rats received 2 mg/kg paclitaxel intraperitoneally. Two weeks after paclitaxel administration, pioglitazone was administered orally at doses of 10 and 20 mg/kg and continued for further two weeks. One group of paclitaxel-naïve rats received vehicle (age-matched control group) and another group of paclitaxel-naïve rats were administered pioglitazone (*per se*; 20 mg/kg, p.o.). The response to behavioral nociceptive tests was assessed on days 0 (before administration of paclitaxel), 7, 14 (on the day before initiation of the treatment), 21, and 28. All the animals were sacrificed after behavioural testing to measure markers of pro-inflammatory cytokine and nitroso-oxidative stress on day 28.

2.5 Behavioral Test Paradigm

2.5.1 Assessment of thermal hyperalgesia

The response to noxious thermal stimulus was determined using a plantar test apparatus (Ugo Basile, Italy) as described by Bijjem et al. [16]. In brief, rats were placed individually in Plexiglas cubicles mounted on a glass surface maintained at $25 \pm 0^\circ\text{C}$. After acclimatization, a thermal stimulus in the form of radiant heat emitted from a focused projection bulb, which was located under the glass floor, was focused onto the plantar surface of the left hind paw and the latency to the first sign of paw licking or withdrawal response to avoid heat pain was taken as an index of pain threshold. Paw withdrawal latencies (PWLs) were recorded at interval of 10 min and the mean of the three values was used for analysis. The intensity of radiant heat was adjusted to give 18 – 19 sec withdrawal latency in rats. A cut-off latency of 20 sec was set to avoid tissue damage.

2.5.2 Assessment of mechanical allodynia

The mechanical threshold for nociceptive flexion was determined by measuring the paw withdrawal threshold (PWT) elicited by stimulation of the left hind paw using Dynamic

Plantar Aesthesiometer (Ugo Basile, Italy) as described by Bijjem et al. [16]. This device generates a mechanical force that increases linearly with time. The maximum force applied by a von Frey type-filament was set 50 g. The nociceptive threshold was defined as the force at which the animal withdrew its paw. In brief, each animal was placed in a test cage with a wire mesh floor, and the tip of a von Frey-type filament was applied to the middle of the plantar surface of the hind paw. Brisk foot withdrawals in response to tactile stimulation were recorded. PWT was expressed as threshold level in grams. Each time the test was repeated three times, and the mean values represented the threshold of the individuals. The decrease in PWT in paclitaxel treated rat indicates mechanical allodynia.

2.6 Collection of Tissues Samples

In this study, at the end of treatment schedule on day 28, the animals were euthanized by overdose of thiopental sodium (200 mg/kg, i.p.) immediately after behavioral assays, followed by collection of lumbar dorsal root ganglia (DRG) and spinal cord for estimation of markers of inflammation and oxidative stress. The L4-L6 DRGs were detected within their intervertebral foramina after total laminectomy and foraminotomy, carefully removed, and stored at -20°C in phosphate buffer pH 7.0. Spinal cord was collected by excising lumbosacral region of spinal cord with L4-L6 segments as the epicenter and immediately kept at 4°C . It was washed in normal saline and weighed. DRGs and one portion of spinal cord were separately homogenized in homogenization buffer containing protease inhibitor. These samples were cold centrifuged and the supernatant was used for estimation of pro-inflammatory cytokine as per manufacturer's specifications. The remaining part of spinal cord was washed with ice cold sterile normal saline, weighed separately, homogenized in ice cold phosphate buffer pH 7.0 and centrifuged for 15 min at 2000g to obtain the clear supernatant for the estimation of oxidative stress markers.

2.7 Pro-inflammatory Cytokine

Pro-inflammatory cytokine, TNF- α , concentration was 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 kit at 450 nm and are expressed as pg per mg tissue.

2.8 Markers of Oxidative Stress

2.8.1 Lipid peroxidation

Lipid peroxidation in spinal cord was estimated colourimetrically by measuring thiobarbituric acid reactive substances (TBARS) by the method of Niehaus and Samuelson [17]. A 0.1 ml of supernatant of spinal cord homogenate was treated with 2 ml of (1:1:1 ratio) thiobarbituric acid (0.37%)-trichloroacetic acid (15%)-hydrochloric acid (0.25 N) reagent and placed in hot water bath for 15 min, cooled and centrifuged and then clear supernatant was measured at 532 nm (UV-1700 Spectrophotometer, Shimadzu, Japan) against blank. Finally, the values are expressed as nmol per g tissue.

2.8.2 Superoxide dismutase

Superoxide dismutase (SOD) activity in spinal cord was measured according to a method described by Misra and Fridovich (1972), by following spectrophotometrically (at 480 nm) the autooxidation of epinephrine at pH 10.4 [18]. In this method, supernatant of the tissue was mixed with 0.8 ml of 50 mM glycine buffer, pH 10.4 and the reaction was started by the addition of 0.02 ml (-)-epinephrine. After 5 min, the absorbance was measured at 480 nm (UV-1700 Spectrophotometer, Shimadzu, Japan). The activity of SOD was expressed as U/g tissue.

2.8.3 Nitrite levels

The spinal cord nitrite levels were measured by the Griess reaction [19]. A 0.1 ml of supernatant was mixed with 250 μ l of 1% sulfanilamide (prepared in 3N HCl) and 250 μ l of 0.1% N-naphthylendiamine with shaking. After 10 min, 1.4 ml of water was added and absorbance was measured at 545 nm (UV-1700 Spectrophotometer, Shimadzu, Japan). The results are expressed as nmol per g tissue.

2.9 Statistical Analysis

The results are presented as mean \pm S.E.M. for at least six animals per group. The data were analyzed by one-way ANOVA (Sigma Stat Version 2.0, SPSS Inc., Chicago, IL, USA) and

the significance of the differences between groups was analyzed by Tukey's test. $P < 0.05$ was considered as statistically significant.

3. RESULTS

3.1 Paclitaxel Injection and Induction of Neuropathy

The baseline paw withdrawal responses (PWLs and PWTs) in each test obtained on day 0 for each rat were relatively stable and showed no significant difference. In age-matched vehicle control animals, the PWLs and PWTs to thermal and mechanical stimulation were similar on all the days of behavioral testing throughout the observation period. There was gradual decline in PWLs and PWTs in paclitaxel administered rats and responses were significantly reduced compared to that of vehicle treated age-matched control rats from day 7 onwards, and remain stable between days 14 and 28 after paclitaxel injection clearly demonstrating the time-dependent development as well as maintenance of steady-state thermal hyperalgesia and mechanical allodynia (Figs. 1 and 2).

3.2 Effect of Paclitaxel on Body Weight

All the groups of rats with similar age and body weight were selected the beginning of the study. All the groups of rats gained normal body weight and administration of paclitaxel or pioglitazone *per se* had no effect on weight gain at the end of observation period. Rats administered with pioglitazone (10 or 20 mg/kg, p.o., for two weeks) looked normal and gained body weight gain similar to the other groups of rats on the last day of experimentation (Table 1).

3.3 Effect of Paclitaxel on Locomotor Activity

Administration of saline, paclitaxel, or pioglitazone *per se* did not alter the motor activity scores during the entire study period (on days 7, 14, 21 and 28) as compared to their respective basal scores observed at the beginning of experimentation. Administration of pioglitazone (10 and 20 mg/kg, p.o., for 2 weeks) had no effect on locomotor activity as compared to paclitaxel-treated group (Table 2).

Table 1. Effect of chronic oral administration of pioglitazone on bodyweight in paclitaxel treated rats

Treatment (mg/kg)	Body weight (g)	
	Initial	Final
Control (age-matched)	217.5 ± 13.5	255.7 ± 21.5
Paclitaxel (PAC) treated	228.2 ± 11.7	264.4 ± 19.3
PIO <i>per se</i> (20)	214.6 ± 12.1	259.4 ± 17.6
PAC + PIO 10	219.9 ± 10.9	250.1 ± 18.7
PAC + PIO 20	223.6 ± 9.6	263.1 ± 21.2

Body weights were measured on day 0 (initial) and day 28 (final). PAC: Paclitaxel; PIO: Pioglitazone. All values are mean ± SEM

Table 2. Effect of chronic oral administration of pioglitazone on locomotor activity in paclitaxel treated rats

Treatment (mg/kg)	Day 0	Day 7	Day 14	Day 21	Day 28
Control	206.1 ± 10.2	216.8 ± 9.9	219.5 ± 12.3	197.7 ± 10.2	213.5 ± 9.9
PAC	196.4 ± 9.7	208.6 ± 10.1	202.9 ± 13.6	212.7 ± 9.4	198.4 ± 11.5
PIO <i>per se</i> 20	201.9 ± 11.9	217.8 ± 12.3	215.8 ± 11.5	193.5 ± 12.2	207.2 ± 11.8
PAC + PIO 10	195.7 ± 14.7	199.4 ± 11.9	209.7 ± 9.9	209.7 ± 10.1	197.9 ± 10.2
PAC + PIO 20	212.3 ± 10.7	214.5 ± 12.7	221.1 ± 14.4	211.7 ± 13.4	217.7 ± 11.8

PAC: Paclitaxel; PIO: Pioglitazone. All values are mean ± SEM

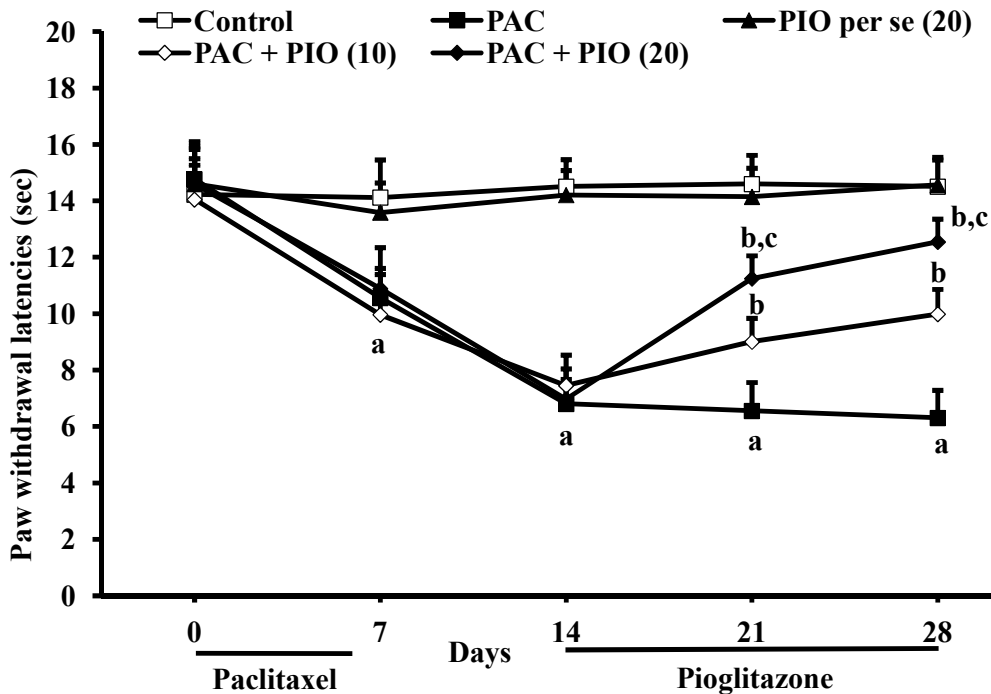


Fig. 1. Effect of repeated oral administration of pioglitazone (PIO; 10 and 20 mg/kg) on thermal paw withdrawal latencies in paclitaxel (PAC) treated rats

Values are expressed as mean ± S.E.M. ^aP < 0.05 vs Control; ^bP < 0.05 vs PAC group; ^cP < 0.05 vs PAC + PIO (10) group.

3.4 Effect of Pioglitazone on Thermal Hyperalgesia

Pioglitazone (*per se*; 20 mg/kg, for 2 weeks) had no effect on paw withdrawal responses to thermal stimulation in paclitaxel-naïve rats. Repeated oral administration of pioglitazone (10 or 20 mg/kg, for 2 weeks) significantly reduced paclitaxel-induced thermal hyperalgesia, both the existing and established thermal hypersensitivities, as compared to paclitaxel-treated rats on days 21 and 28. At the end of study period, high dose pioglitazone (20 mg/kg for two weeks) significantly improved paclitaxel-induced thermal hypersensitivity as compared to that of low dose pioglitazone (10 mg/kg for two weeks) (Fig. 1).

3.5 Effect of Pioglitazone on Mechanical Allodynia

Administration of pioglitazone (*per se*; 20 mg/kg, p.o., for two weeks) did not alter paw withdrawal responses to mechanical stimulation as compared to basal responses during the entire observation period in paclitaxel-naïve rats. However, pioglitazone (10 or 20 mg/kg, p.o., for 2 weeks) significantly increased PWTs in paclitaxel treated rats indicates marked reversal

of existing and established mechanical allodynia on days 21 and 28. Furthermore, on day 28, high dose pioglitazone (20 mg/kg, p.o., for two weeks) showed prominent effect on reversal of paclitaxel-induced allodynia as compared to that of low dose pioglitazone (10 mg/kg, p.o., for two weeks) (Fig. 2).

3.6 Effect of Pioglitazone on Pro-inflammatory Cytokine in DRG and Spinal Cord

There was a significant increase in the pro-inflammatory cytokine, TNF- α , levels in lumbar DRGs and spinal cord in paclitaxel injected rats when compared to that of age-matched control rats on day 28. Administration of pioglitazone (*per se*; 20 mg/kg, p.o., for 2 weeks) had no effect on TNF- α levels in both the DRGs and the spinal cords of in paclitaxel-naïve rats. On the other hand, similar administration of pioglitazone (10 or 20 mg/kg p.o., for 2 weeks) significantly reduced the levels of TNF- α in both the DRGs and the spinal cords in paclitaxel injected rats and high dose pioglitazone (20 mg/kg) showed significant reduction in TNF- α levels at both the sites as compared to low dose pioglitazone (10 mg/kg) (Fig. 3).

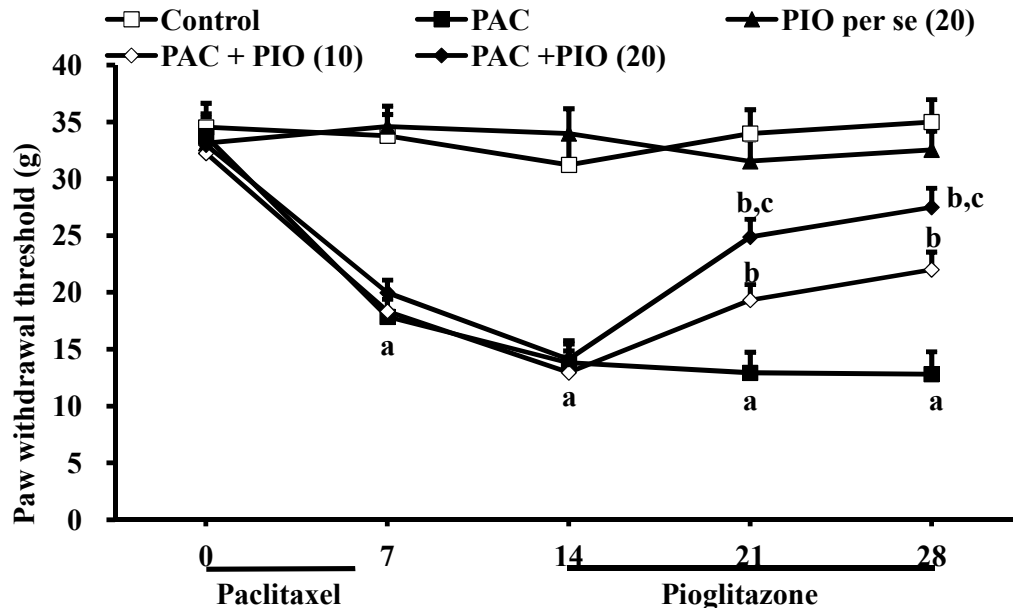


Fig. 2. Effect of repeated oral administration of pioglitazone (PIO; 10 and 20 mg/kg) on mechanical paw withdrawal threshold in paclitaxel (PAC) treated rats
 Values are expressed as mean \pm S.E.M. ^aP < 0.05 vs Control; ^bP < 0.05 vs PAC group; ^cP < 0.05 vs PAC + PIO (20) group.

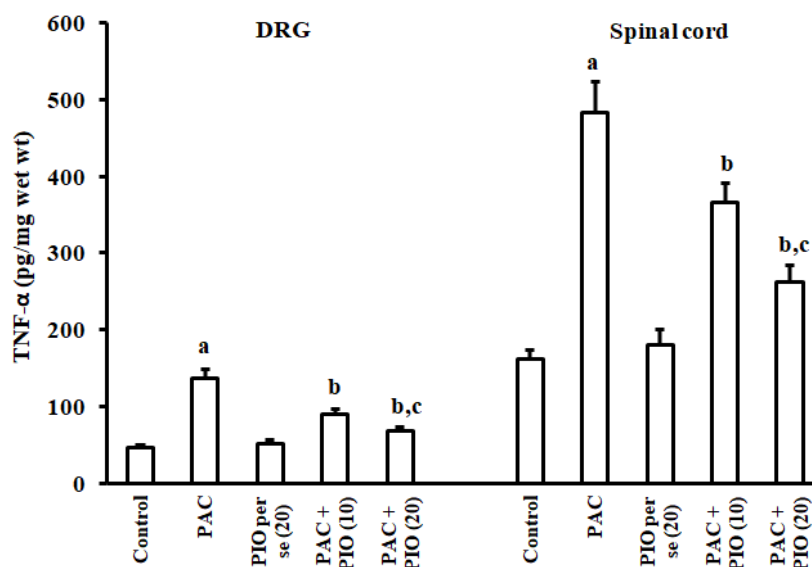


Fig. 3. Effect of repeated oral administration of pioglitazone (PIO; 10 and 20 mg/kg) on tumour necrosis factor-alpha (TNF- α) in lumbar dorsal root ganglia (DRG) and spinal cord

All values are expressed as mean \pm S.E.M. ^aP < 0.05 vs Control; ^bP < 0.05 vs PAC group; ^cP < 0.05 vs PAC + PIO (10) group.

Table 3. Effect of chronic oral administration of pioglitazone on the markers of oxidative and nitrosative stress in spinal cord of paclitaxel treated rats

Treatment (mg/kg)	TBARS (nmol/g tissue)	SOD (U/g tissue)	Nitrite (nmol/g tissue)
Control (age-matched)	51.2 \pm 7.1	33.1 \pm 3.8	101.8 \pm 8.9
Paclitaxel treated	173.5 \pm 14.9 ^a	8.5 \pm 0.9 ^a	211.5 \pm 14.7 ^a
PIO per se (20)	59.29 \pm 9.7	31.8 \pm 2.1	105.7 \pm 8.7
PAC + PIO 10	119.8 \pm 10.4 ^b	18.1 \pm 1.5 ^b	154.5 \pm 12.6 ^b
PAC + PIO 20	79.8 \pm 8.3 ^{b,c}	26.3 \pm 1.3 ^d	120.7 \pm 10.3 ^{b,c}

PAC: Paclitaxel; PIO: Pioglitazone; TBARS: Thiobarbituric acid reactive substances; SOD: Superoxide dismutase; TNF- α : Tumour necrosis factor – alpha. All values are mean \pm SEM; ^aP < 0.05 vs Control; ^bP < 0.05 vs PAC group; ^cP < 0.05 vs PAC + PIO 10 group.

3.7 Effect of Pioglitazone on the Markers of Oxidative and Nitrosative Stress

Paclitaxel significantly elevated TBARS and nitrite levels, the marker of oxidative and nitrosative stress accompanied by marked reduction in the activity of SOD, an antioxidant defense enzyme in spinal cord. Administration of pioglitazone (10 and 20 mg/kg, for two weeks) significantly diminished paclitaxel-induced nitroso-oxidative stress. Conversely, pioglitazone (*per se*; 20 mg/kg, p.o., for 2 weeks) had no effects on the markers of oxidative and nitrosative stress in paclitaxel-naïve rats as compared to age-matched control group. Remarkably, high dose pioglitazone (20 mg/kg) showed significant reduction in nitroso-oxidative

stress as compared to low dose pioglitazone (10 mg/kg) (Table 3). In addition, paclitaxel injection significantly decreased the activity of SOD in spinal cord indicating that paclitaxel could disrupt the antioxidant defense systems in the spinal cord. Administration of pioglitazone (10 and 20 mg/kg for two weeks) prominently restored the activity of SOD in spinal cord with more marked effect was noticed with the high dose pioglitazone (20 mg/kg) (Table 3).

4. DISCUSSION

The present results show that administration of pioglitazone, a PPAR- γ agonist reduced paclitaxel-induced neuropathic hypersensitivity. This suggests that PPAR- γ activation is

necessary for the antihyperalgesic and antiallodynic effects of pioglitazone to occur. These antinociceptive effects may be expected as pioglitazone has been shown to be a selective and potent PPAR- γ agonist and to cross blood-brain-barrier readily to activate PPAR- γ and modulate central nociceptive processing [13,15,20]. Indeed, the present study shows that pharmacological activation of PPAR- γ is the only factor responsible for antinociceptive effects that is independent of any behavioral and developmental changes due to paclitaxel. In fact, similar to paclitaxel, pioglitazone *per se* had no effect on body weight gain and did not affect spontaneous motor activity. This provides evidence that the observed effects of pioglitazone in modulating paclitaxel-induced mechanical allodynia and thermal hyperalgesia during maintenance are potentially due to PPAR- γ activation, but not any effect on general body weight and behavioral changes. Additionally, it is observed that pioglitazone attenuated established paclitaxel-induced neuropathic pain state. It is also observed that the basal paw withdrawal responses were not affected by pioglitazone in paclitaxel-naïve animals revealing that the potential antinociceptive effects of pioglitazone in paclitaxel-induced neuropathy are not because of hypoalgesia. Most importantly, pioglitazone penetrates blood-brain-barrier and had shown anti-inflammatory effects after systemic administration [13,21,22]. The present results are consistent with previous reports wherein PPAR- γ activation showed antinociceptive and anti-inflammatory effects following spinal cord, peripheral nerve injury, diabetes, and cisplatin-induced neuropathic pain [13,15,20,23]. Within the possible limits, the present experimental design was not able to explain any potential additional effect. Thus, these results suggest that PPAR- γ activation plays a major role in antinociceptive effect of pioglitazone in alleviating paclitaxel-induced neuropathic pain and provides compelling evidence of potential antinociceptive effects of PPAR- γ activation.

Evidence points to important roles for immune cells, immune reactions, and pro-inflammatory cytokines and inflammatory mediators in generating not only inflammatory pain but chronic, neuropathic pain as well. Though neurotoxic and peripheral neuronal damage has been implicated as possible mechanisms for paclitaxel-induced neuropathic pain; however, emerging evidence indicates involvement of inflammation in both the periphery and the CNS

in the development and maintenance of neuropathic pain [24-26]. In line with this, we observed elevated TNF- α levels in both lumbar DRG and spinal cord further supporting prominent role of both peripheral and spinal inflammation in paclitaxel-induced neuropathic pain. It has been reported that a sequence inflammatory events starting from the activation of Toll-like receptor 4, fractalkine and its receptors, increased expression of MCP-1 in DRG, and finally to infiltration of macrophages occurs following paclitaxel [7,27]. Moreover, up-regulation of chemokines, the time course of DRG infiltration of macrophages matches the onset of peripheral epidermal nerve fiber loss and development of paclitaxel-induced pain hypersensitivities [7,27,28]. In the present study, pioglitazone markedly reduced the level of TNF- α in lumbar DRGs supporting activation of PPAR- γ in the periphery. It is reported that PPAR- γ activation reduced macrophages, macrophage-driven neuroinflammation, inflammatory cytokines, and chemokines in the periphery and improved behavioral symptoms of neuropathic pain due to nerve injury and diabetes [29-31]. Moreover, PPAR- γ -deficient macrophages showed an increased production of pro-inflammatory cytokines TNF- α , IL1- β , IL-6, and IL-12 upon long-term lipopolysaccharide stimulation indicating that PPAR- γ is essential for reducing release of pro-inflammatory cytokines [32]. Further, PPAR- γ activation down-regulated interferon- γ -induced gene expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) and attenuated the chemotactic response to MCP-1 [29,30]. Indeed, nuclear PPAR- γ receptors present in the neurons of the DRGs and the dorsal horn of the spinal cord which are the sites for nociceptive processing [31]. Thus, activation of PPAR- γ receptors contributed to anti-inflammatory effects of pioglitazone, a PPAR- γ agonist, thereby reducing macrophage infiltration and prevented release of pro-inflammatory cytokines in the DRGs.

Recent studies demonstrated paclitaxel-induced activation of non-neuronal immune cells, microglia and astrocytes, and subsequent increase in expression of phosphorylated NF- κ B, and release of chemokines, IL-1 β , IL-10, IL-20, and TNF- α peripherally in sciatic nerve and lumbar DRG as well as centrally in spinal cord [8,9]. Evidence exists that peripherally released chemokines and cytokines dysregulate the blood-spinal cord permeability and evoke the influx of inflammatory mediators into spinal cord

[25,33]. It is obvious explanation wherein increase in TNF- α in lumbar DRGs parallels with that of spinal cord noticed in our study strongly supports neuro-immune communication and activation of spinal immune cells. In the present study, pioglitazone administration reduced elevated level of TNF- α in DRGs and spinal cord that was parallel with the alleviation of established allodynia and hyperalgesia. The centrally mediated effects of PPAR- γ activation have also been reported wherein several PPAR- γ agonists suppressed microglia and astrocytes activation, reduced the development of spinal neuroinflammation, and showed antinociceptive and neuroprotective effects [14,15,34,35]. A recent study showed decreased transcription factor in parallel with reduced expression of PPAR- γ in spinal cords after paclitaxel administration. In the same study, it is also observed that administration of probiotic formulation, SLAB51 reduced serum cytokine levels and increased PPAR- γ protein levels in spinal cord [36]. In accordance with previous studies, it seems activation of PPAR- γ relieved neuropathic pain state, possibly through an inhibitory action on neuro-immune glial activation and subsequent TNF- α release in spinal cord.

Several lines of evidence implicated the involvement of free radicals and subsequent oxidative stress in peripheral and central sensitization in neuropathic pain states [37,38]. Indeed, paclitaxel induces biochemical changes in mitochondria of sensory axons of DRG and disrupt their functions leading to the increased generation of ROS, reactive nitrogen species, and decreased antioxidant defenses [10,38-40]. Moreover, paclitaxel induces NADPH oxidase-derived ROS in neurons, which may lead to direct neuronal damage and neuronal hyperexcitation [39,41]. Hyperexcitation of non-inflammatory cells, such as neurons and activation of non-neuronal cells, such as microglia and astrocytes stimulate production of intracellular ROS, which act as a second messenger in several signaling pathways, including those involved in triggering the inflammatory response and the migration of inflammatory cells into the lesioned area [41-43]. Cross-talk between nitroso-oxidative stress and nociceptive processing as well as neuroprotection in both the periphery and the CNS has also been observed in several models of neuropathic pain and neurodegeneration [24,37,38]. This interaction has recently been investigated in CINP including paclitaxel-induced neuropathic pain [10,38,39]. Indeed, various

strategies targeting oxidative stress by using antioxidants reduced the development and maintenance of paclitaxel-induced hyperalgesia and allodynia [10,38-40]. Consistent with previous reports, paclitaxel showed increased nitroso-oxidative stress that parallel with the peaked steady-state neuropathic hypersensitivity. We noticed that pioglitazone markedly reduced nitroso-oxidative stress and improved depleted antioxidant enzyme SOD activity in the spinal cord following paclitaxel administration. It is also observed that pioglitazone had not altered nitroso-oxidative stress in paclitaxel-naïve rats indicating the antioxidant effects of pioglitazone are due to activation of PPAR- γ only in neuropathic state. Moreover, activation of PPAR- γ had shown antioxidant potential by reducing lipid peroxidation, and improving antioxidant defenses [23,44,45]. Thus, the results are explicable that activation of PPAR- γ by pioglitazone reduced nitroso-oxidative stress and restored antioxidant defenses, and thereby alleviated paclitaxel-induced neuropathic state.

Accumulating data indicates pleiotropic effects of PPAR- γ activation contributed to neuroprotective and anti-inflammatory effects of PPAR- γ agonists. It is reported that dysregulated expression of nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1(HO-1) parallels with downregulation of PPAR- γ in the spinal cords of paclitaxel treated rats [40,46]. It is plausible that such mechanisms might also be involved in reducing the development and maintenance of CINP. Of note, endogenous cellular antioxidant pathways which include Nrf2, HO-1, Nrf2-induced target genes in neurons, DRGs, spinal cord microglia and astrocytes are shown to be increasingly upregulated due to PPAR- γ activation [46]. In addition, pioglitazone is reported to act through nongenomic PPAR- γ mechanisms in reducing nerve injury-induced neuropathic pain [14]. Compelling evidence exist on PPAR- γ agonists as possible antinociceptive agent along with the results of the present study further supports the therapeutic potential of PPAR- γ activation in attenuating CINP.

5. CONCLUSION

The results of the present study reveal that repeated administration of pioglitazone, a PPAR- γ agonist showed antiallodynic and antihyperalgesic effects against paclitaxel-induced neuropathic hypersensitivity and that the antinociceptive effect is mediated by PPAR- γ

activation. Furthermore, the results also showed that pioglitazone exerted anti-inflammatory and antioxidant effects in reducing paclitaxel-neuropathic pain associated neuroimmune inflammation and oxidative stress. These results suggest that pioglitazone acts by a double mechanism that involves a pharmacological PPAR- γ activation leading to decreased TNF- α , a pro-inflammatory cytokine in both the DRGs and the spinal cord as well as reduced nitroso-oxidative stress in the spinal cord and thereby attenuating paclitaxel-induced behavioral hypersensitivity. Our findings identify pharmacological activation of PPAR- γ as a promising molecular and therapeutic target in CIPN and provide rationale for the clinical evaluation.

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 (OQNP/PCS/0815/FW/12) and study was performed in accordance with the guidelines for Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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