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ORIGINAL ARTICLE

# Effect of dipyrone and thalidomide alone and in combination on STZ-induced diabetic neuropathic pain

**Neha Chauhan · Rajeev Taliyan · Pyare Lal Sharma**

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**Abstract** Diabetic neuropathy is recognized as one of the most common complications of chronic diabetes, but its pathophysiological mechanism is complex and yet to be completely explored. Monotherapy with conventional analgesics fails to provide adequate pain relief in peripheral diabetic neuropathy. There are a number of evidence suggesting that tumor necrosis factor (TNF-α) plays an important role in the pathogenesis of peripheral diabetic neuropathy. TNF-α up-regulation activates nuclear factor κB, which further up-regulates cyclooxygenase (COX)-2 leading to altered prostaglandin profile. Inhibition of TNF-α and COX-2 provides beneficial effect on diabetic neuropathy by decreasing the oxidative stress level and by preventing neuronal hypersensitivity due to an increased prostaglandin level. The present study was designed to assess the effect of dipyrone and thalidomide on streptozotocin (STZ)-induced neuropathic pain behavior in rats. STZ 50 mg/kg, i.p. was administered to induce experimental diabetes in the rats. Three weeks following STZ, dipyrone (300 and 600 mg/kg, i.p.) and thalidomide (25 and 50 mg/kg, i.p.) alone and subeffective dose combination of dipyrone and thalidomide (300 and 25 mg/kg$^{-1}$, i.p.) administered daily for 2 weeks significantly attenuated thermal hyperalgesia, mechanical allodynia, and formalin-induced phase-2 flinching response. Moreover, the subeffective dose combination of dipyrone and thalidomide and preemptive treatment with thalidomide (50 mg/kg) reduces oxidative stress in diabetic rats. In conclusion, the combination of subeffective dose of dipyrone and thalidomide prevented the development and maintenance of experimental diabetic neuropathy. The combination of thalidomide (TNF-α inhibitor) and dipyrone (COX inhibitor) may be used as a potential therapeutic agent for the treatment of diabetic neuropathy.

**Keywords** Diabetic neuropathy · Hyperalgesia · Allodynia · Tumor necrosis factor · Cyclooxygenase

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## Introduction

One of the most common chronic complications of diabetes mellitus is diabetic neuropathy, but it remains probably the least understood complications (Greene et al. 1997), which is mainly characterized by spontaneous pain, abnormal sensations such as paresthesia, allodynia (pain responses to innocuous stimuli), and hyperalgesia (exaggerated pain responses to noxious stimuli). The contribution of hyperglycemia in pathogenesis of diabetic neuropathy is beyond controversy, which eventually leads to accumulation of advanced glycation end-products (Brownlee 2005), protein kinase C isoform activation, mitochondrial dysfunction (Vinik et al. 2003), and activation of nuclear factor-κB (NF-κB) (Wang et al. 2006). All these pathways converge in the production of oxidative stress.

Reactive oxygen/nitrogen species (Pop-Busui et al. 2006a, b) and inflammatory cytokine tumor necrosis factor α (TNF-α) (Taliyan et al. 2010) play a key role in diabetic neuropathy, starting from the development of the initial stages of diabetes to the progression of the later stages of

neuropathic pain (Taliyan et al. 2011). TNF-α or interleukin (IL) is released by macrophages, Schwann cells, and lymphocytes in diabetic nerves in humans and animals (Conti et al. 2002; Yagihashi et al. 2007). These studies suggest a role of TNF-$\alpha$ in the regulation of development of hyperalgesia and allodynia and apoptosis in diabetic animals, inflammatory or immunological disease. This leads to much effort recently in finding ways to down-regulate its production or inhibit its effects. A number of chimeric TNF-α antibodies such as Adlimumab, Etanercept, and CDP571 have been developed to treat conditions associated with elevated TNF-α, but these antibodies have certain limitations including their high cost and potential adverse effect (Scheinfeld 2004). Thalidomide, a derivative of glutamic acid, inhibits TNF-α synthesis by decreasing the half-life of TNF-α mRNA and was reported to possess various beneficial pharmacological properties including antiinflammatory, immunomodulatory, and antiangiogenic effects (Ribeiro et al. 2000; Ye et al. 2006) and was reintroduced, despite its powerful teratogenic nature, as treatment for diverse chronic immunological/inflammatory diseases, and it is suggested as a promising treatment for neurodegenerative diseases (Sampaio et al. 1991). Thalidomide's immunomodulatory effects and inhibition of the synthesis and release of proinflammatory cytokines as well as increases the release of anti-inflammatory cytokines are based on its capacity to modify T-helper cell phenotype from a proinflammatory Th1 to an anti-inflammatory Th2 pattern, on the basis of the type of cytokines produced (Corrala and Kaplan 1999; Ribeiro et al. 2000; Sommer et al. 2001).

TNF-$\alpha$ activation further regulates the production of additional cytokines and nerve growth factor, macrophage recruitment, myelin removal, regeneration, and neuropathic pain by different mechanisms. In addition, there is simultaneous activation of cyclooxygenase (COX)-2 in the peripheral nerves of STZ diabetic rats (Pop-Busui et al. 2002), contributing to diabetes-induced neuropathic pain. COX-2 up-regulation results in altered prostaglandin profile in which there is an increased production of vasoconstricting prostaglandin H2 (PGH2), thromboxane A2, and prostaglandin F2-alpha (PGF2-α) and reduction in vasodilatory prostacyclin (PGI2). In addition, COX-2 up-regulation increases reactive oxygen species (ROS) generation, which further exacerbates oxidative stress.

Current treatment of peripheral diabetic neuropathy (PDN) involves the use of tricyclic antidepressant, selective serotonin reuptake inhibitors (Mckeage 2007), anticonvulsants, opioids and antioxidant protein kinase C inhibitors, COX-2 inhibitors (Kellog et al. 2008), and nonsteroidal anti-inflammatory drugs as mild analgesics and so on Treatment with these drugs is often limited because of partial effectiveness and side effects associated with these drugs (Chong and Hester 2007; O'Connor 2009). Thus, there is a need of new therapeutic interventions targeting primary mechanisms resulting in nerve damage in PDN.

Dipyrone (COX inhibitor) and thalidomide (TNF-α inhibitor) have been evaluated for efficacy in STZ-induced neuropathic pain in rats. Dipyrone acts as an effective analgesic and antipyretic agent (Ceraso. 1994), exerting its antinociceptive effect by inhibition of prostaglandin synthesis in the peripheral and the central nervous system (Abbate et al. 1990; Shimada et al. 1994), although its precise mechanism of action remains unclear.

Initially, analgesia by dipyrone was explained by an inhibitory action on PG synthesis. However, Nikolova et al. (1980) suggested that the profile of the pharmacological effects of dipyrone is certainly different from that of other nonsteroidal anti-inflammatory drugs. Lorenzetti and Ferreira (1996) have indicated that the involvement of arginine–nitric oxide (NO) pathway in primary sensory neurons contributes to dipyrone-induced spinal and peripheral analgesia. Moreover, it has been reported that the peripheral analgesic effect of dipyrone may result from direct blockade of hyperalgesia rather than from prevention of the release of prostaglandins in inflamed tissues (Lorenzetti and Ferreira 1985). In addition to this, several studies indicated that dipyrone induces an antinociceptive effect both by peripheral and central mechanisms (Akman et al. 1996). Both these drugs have shown efficacy in various inflammatory models. However, there is no study reported on the use of these drugs alone and in combination on STZ-induced neuropathic pain model in rats, which is addressed in the present study.

## Experimental animals

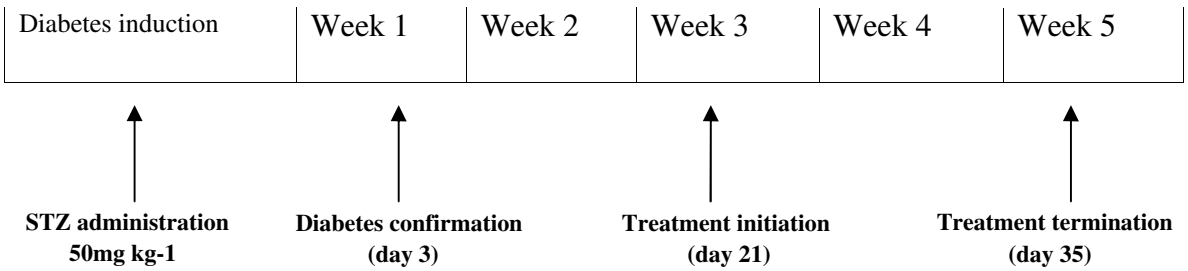
Wistar rats weighing 200–280 g were used for behavioral paradigm of PDN. The experimental protocol was approved by the Institutional Animal Ethics Committee.

## Induction and assessment of diabetes in rats

Experimental diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (50 mg $kg^{-1}$) freshly dissolved in citrate buffer pH. Serum glucose level was assessed by using enzymatic glucose oxidase peroxidase commercially available kit method, 72 h after STZ induction. Only rats with blood glucose concentration more than 240 mg/dl were considered diabetic and used for the study. Body weight and serum glucose were measured before and at the end of the experiment to see the effect of pharmacological interventions on these parameters.

Treatment schedule

All animals were acclimatized to a laboratory environment for at least 2 h before testing




Group I: Saline-treated normal control
Group II: Saline-treated diabetic control
Group III: Positive control: pregabalin (30 mg $kg^{-1}$, i.p./day) for 2 weeks in STZ-induced diabetic rats
Group IV: Dipyrone (300 mg $kg^{-1}$, i.p./day) for 2 weeks in STZ-induced diabetic rats
Group V: Dipyrone (600 mg $kg^{-1}$, i.p./day) for 2 weeks in STZ-induced diabetic rats
Group VI: Thalidomide (25 mg $kg^{-1}$, i.p./day) for 2 weeks in STZ-induced diabetic rats
Group VII: Thalidomide (50 mg $kg^{-1}$, i.p./day) for 2 weeks in STZ-induced diabetic rats
Group VIII: Subeffective dose combination of dipyrone and thalidomide (300 mg $kg^{-1}$ and 25 mg $kg^{-1}$, i.p./day) for 2 weeks in STZ-induced diabetic rats
Group IX: Thalidomide (50 mg $kg^{-1}$, i.p./day) pretreatment, started 1 day before STZ administration and continued up to 5 weeks. Body weight and serum glucose level were measured before and after the last dose of treatment (5 weeks of diabetes induction)

Assessment of thermal hyperalgesia

Hyperalgesia to thermal stimulation was determined using a Plantar Test Apparatus (Ugo Basile, Comerio, Italy) modeled as described by Hargreaves et al. 1988. In brief, rats were placed individually in Plexiglas cubicles mounted on a glass surface maintained at 25°C. 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 hind paw; paw withdrawal latencies (PWLs) were recorded at the interval of 15 min, and the mean of the three values was used for analysis. A cutoff latency of 30 s was set to avoid tissue damage. The response latency was determined using a timer linked to the photodiode motion sensors in the plantar reflex device.

Assessment of mechanical allodynia

The threshold for touch sensitivity was measured in the hind paws using an automated apparatus for applying reproducible light touch (Dynamic plantar Aesthesiometer 37400-002; Ugo Basile). Animals were placed in their compartments on the metal mesh surface allowed to adjust for at least 20 min. After positioning the filament of touch stimulator unit, beneath the selected hind paw of the animal, the unit is started and electrodynamic actuator lifts the stainless steel filament exerting an upward force. The force increases until the animal moves its paw or until the point at which greatest present force is met. The maximum value of force in grams (50 g) was previously fixed (Arevalo et al. 2004).

Assessment of formalin-induced flinching in rats

Formalin-induced flinching behind was assessed as described by Calcutt et al. (1996) and was followed for the rat formalin test. Age-matched control and diabetic animals received subcutaneous injection of 50Pl of 2% formalin solution in normal saline to the plantar surface of the right hind paw. The animals were transferred to an observation chamber constructed to allow continuous visualization of the paws. The number of flinches was counted in a 5-min interval for the next 60 min. Two phases of spontaneous flinches were observed after formalin injection separated by a quiescent phase. Phase I was defined as the initial measurement of flinching (0–10 min after formalin injection), quiescent phase as a measurement made at

10–20 min, and phase II as all the subsequent measurements after formalin injection. The results are expressed as the sum of flinching responses in phases 1 and 2 of the formalin test.

### Collection of blood and tissue samples in rats

In this study, at the end of treatment schedule on day 35, blood was collected for serum glucose estimation, and the animals were euthanized by cervical dislocation immediately after behavioral assays, followed by collection of sciatic nerve for estimation of markers of oxidative stress, and sciatic nerves were rapidly removed, washed with sterile normal saline, and weighed. A 10% (wt/vol) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4) and centrifuged for 15 min at 2,000×*g* to obtain the clear supernatant for the estimation of oxidative stress markers.

### Biochemical assessment

#### *Estimation of lipid peroxidation*

Lipid peroxidation in the sciatic nerve was estimated colorimetrically by measuring thiobarbituric acid reactive substances by the method of Niehius and Samuelsson (1968). Supernatant (0.1 ml) of sciatic nerve 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 a blank solution. Finally, the values are expressed as nanomoles per gram of tissue.

#### *Estimation of reduced glutathione*

The concentration of endogenous antioxidant-reduced glutathione (GSH) level in the sciatic nerve was estimated following the method described by Lou et al. (1988). In this method, 0.2 ml of supernatant was mixed with 1.78 ml of 1.0 M Tris buffer (pH 8.2) with 0.02 M ethylenediaminetetrachloroacetic acid. Then, 20PL of 0.1 M 5,5′-dithio-bis-2-nitrobenzoic acid (Ellman's reagent) was added to the mixture, and absorbance was noted at 412 nm (UV-1700 Spectrophotometer, Shimadzu); the values are expressed as picomoles per gram of tissue.

#### *Measurement of nitrite*

The nitrite concentration in the serum was measured by Griess reaction (Sastry et al. 2002). In this method, 0.1 ml of supernatant of the nerve homogenate was mixed with 0.25 ml of 1% sulfanilamide (prepared in 3 N HCL) and 0.25 ml of 0.1% *N*-(1-naphthyl) ethylenediaminedihydrochloride with shaking. After 10 min, absorbance was measured at 545 nm (UV-1700 Spectrophotometer; Shimadzu), and the values of nitrite concentration were obtained from sodium nitrite standard curve and are expressed in nanomoles per gram of tissue.

### Drugs and chemicals

Thalidomide, dipyrone, pregabalin, streptozotocin (STZ; Sigma Aldrich Corporation, Bangalore, India) and formalin (37% formaldehyde) (SD Fine Chemicals, Mumbai, India) were used in this study. Glucose oxidase peroxidase estimation kit was purchased from Erba, Transasia Bio-Medicals, Mumbai, India. Unless stated, all other chemicals and biochemical reagent of highest analytical grade quality were used. Dipyrone for i.p. administration was freshly prepared by solubilizing in sterile normal saline. Thalidomide for i.p. administration was dissolved in 10% dimethylsulfoxide. Formalin was diluted with sterile normal saline. Dose of dipyrone (Hernandez-Delgadillo and Cruz 2004), and others were selected on the basis of a previous report that was replicate by a pilot study ($n=3$).

### Statistical analysis

The results are expressed as mean±SD. The behavioral data were analyzed using two-way analysis of variance followed by between-group differences by Bonferroni post hoc test for multiple comparison. $p<0.05$ was considered statistically significant.

## Results

### Effect of dipyrone and thalidomide alone and in combination on body weight and on serum glucose level

Rats injected with STZ (50 mg/kg, i.p.) showed a significant rise in serum glucose and a significant decline in body weight (Table 1), as compared to age-matched normal control rats (vehicle treated). Monotherapy with dipyrone and thalidomide and subeffective dose combination of (dipyrone and thalidomide) in STZ diabetic rats did not alter the 5-week diabetic hyperglycemia and reduced body weight, as compared to vehicle-treated diabetic rat. Furthermore, pretreatment with thalidomide (50 mg $kg^{-1}$, i.p.) also did not affect hyperglycemia in diabetic rats and their body weight.

### Behavioral assessment

#### *Effect of dipyrone and thalidomide alone or in combination on thermal hyperalgesia*

The nociceptive threshold was significantly reduced in diabetic control rats compared to normal control rats (Fig. 1). In

**Table 1** Effect of dipyrone and thalidomide alone or in combination on body weight and blood glucose level in rats

| Treatment (mg kg$^{-1}$) | Body weight (g) | | Blood glucose (mg dl$^{-1}$) | |
|---|---|---|---|---|
| | Initial | Final | Initial | Final |
| NC | 227.33±14.03 | 256.5±17.98 | 108.86±4.18 | 108.38±10.19 |
| DC | 260.66±29.64 | 186.83±23.41* | 110.34±3.92 | 487±18.70* |
| D + P 30 | 256.83±16.43 | 191.83±17.67 | 110.16±4.708 | 477±19.01 |
| D + DPN 300 | 204±26.31 | 164.8±16.05 | 15.96±4.48 | 461±29.81 |
| D + DPN 600 | 266.8±10.03 | 185.2±28.46 | 110.6±4.03 | 446.2±24.65 |
| D + TH 25 | 235±9.71 | 142.66±18.83 | 109.5±4.50 | 412.16±18.33 |
| D + TH 50 | 32.5±12.78 | 153±26.19 | 111.33±2.8 | 426.16±16.82 |
| D + DPN 300 + TH 25 | 236.2±10.51 | 131.8±11.10 | 107.8±3.86 | 473.44±17.33 |
| D + TH 50 (pre) | 234.66±9.77 | 131.16±9.96 | 110.16±2.63 | 419±18.27 |

Values are mean±SD

*NC* normal control; *DC* diabetic control; *D + P 30* pregabalin-treated diabetic rats; *DPN + D 300 and D + DPN 600* dipyrone-treated diabetic rats; *D + TH 25 and D + TH 50* thalidomide-treated diabetic rats; *D + DPN 300 + T H 25* dipyrone and thalidomide combination-treated diabetic rats; *D + TH 50 (pre)* pretreatment with thalidomide in diabetic rats

*$p<0.05$ vs. normal control

this study, monotherapy with subeffective dose of dipyrone (300 mg kg$^{-1}$, i.p.) and thalidomide (25 mg kg$^{-1}$, i.p.) for 3 weeks had no effect on PWLs in age-matched diabetic rats, whereas monotherapy with a high dose of dipyrone (600 mg kg$^{-1}$, i.p.) and thalidomide (50 mg kg$^{-1}$, i.p.) significantly attenuated the development of thermal hyperalgesia in diabetic rats compared to untreated diabetic rats. Moreover, treatment with subeffective dose combination of dipyrone

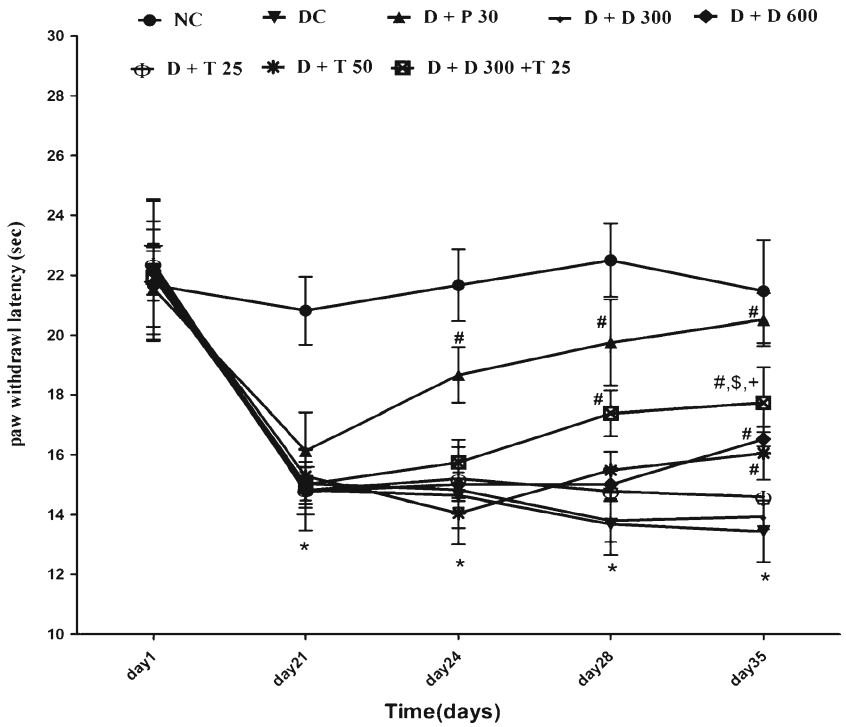



**Fig. 1** Effect of dipyrone and thalidomide alone or in combination on thermal hyperalgesia, in control and STZ-injected diabetic rats. Values are expressed as mean±SD. $n=6$. *$p<0.05$ vs. normal control; $^{\#}p<0.05$ vs. diabetic control; $^{\$}p<0.05$ vs. dipyrone (300 mg kg$^{-1}$); $^{+}p<0.05$ vs. thalidomide (25 mg kg$^{-1}$). *NC* normal control; *DC* diabetic control; *D + P30* pregabalin (30 mg kg$^{-1}$)-treated diabetic group; *D + D 300 and D + D 600* dipyrone (300 and 600 mg kg$^{-1}$)-treated diabetic groups; *D + T 25 and D + T 50* thalidomide (25 and 50 mg kg$^{-1}$)-treated diabetic group; *D + D 300 + T 25* dipyrone (300 mg kg$^{-1}$) and thalidomide (25 mg kg$^{-1}$) combination-treated diabetic group. *Arrow* indicates day of initiation of treatment

(300 mg $kg^{-1}$, i.p.) and thalidomide (25 mg $kg^{-1}$, i.p.) produced a significant increase in pain threshold, as evident from the increase in the PWL on days 28 and 35. In thalidomide (50 mg $kg^{-1}$) preemptive study paradigm, there was a time-dependent increase in PWLs, but a significant difference in latency was seen only on 35 days (Fig. 2). Moreover, pregabalin (30 mg $kg^{-1}$)-treated rat used as a positive control showed a significant time-dependent increase in pain latency on days 24, 28, and 35, as compared to untreated diabetic rats.

*Effect of effect of dipyrone and thalidomide alone and in combination on mechanical allodynia*

Diabetic animals showed a significant decline in the paw withdrawal threshold in Dynamic Plantar Asthesiometer Test, as compared to the age-matched normal control rats, indicating development of mechanical allodynia. Monotherapy with dipyrone and thalidomide at subeffective dose (dipyrone 300 mg $kg^{-1}$ and thalidomide 25 mg $kg^{-1}$, i.p,) failed to demonstrate any significant effects on paw withdrawal threshold except at higher doses (dipyrone 600 mg $kg^{-1}$ and thalidomide 50 mg $kg^{-1}$). Treatment with subeffective dose combination of dipyrone (300 mg $kg^{-1}$, i.p.) and thalidomide (25 mg $kg^{-1}$, i.p.) significantly increased pain threshold as evident from the increase in the paw withdrawal threshold on days 24, 28, and 35, as compared to untreated diabetic animal (Fig. 3). In thalidomide (50 mg $kg^{-1}$) preemptive study paradigm, there was an increase in paw withdrawal threshold, but a significant effect was observed on 28 and 35 days (Fig. 4). Pregabalin-treated animal (30 mg $kg^{-1}$, i.p.) showed a significant time-dependent increase in pain threshold on days 24, 28, and 35, as compared to untreated diabetic rats.

*Effect of pharmacological intervention on formalin-induced flinching behavior in diabetic rats*

No significant difference in the sum of flinches counted in phase 1 was observed between age-matched normal control, diabetic control, and dipyrone- and thalidomide-treated groups on day 35 (Fig. 5). However, significant enhancement of phase 2 flinching responses was observed during the time course of the formalin test, resulting in a state of hyperalgesia on day 35 in the diabetic control group. No significant difference in the sum of flinches counted in phases 1 and 2 was observed in monotherapy with subeffective dose of dipyrone (300 mg $kg^{-1}$) and thalidomide (25 mg $kg^{-1}$) in diabetic rats. However, in dipyrone (600 mg $kg^{-1}$) and subeffective dose combination group of dipyrone (300 mg $kg^{-1}$) and thalidomide (25 mg $kg^{-1}$), flinching behavior was significantly attenuated as compared to untreated diabetic rats. Preemptive thalidomide (50 mg $kg^{-1}$) and positive control pregabalin (30 mg $kg^{-1}$, i.p.)-treated rats showed a significant decrease of phase 2 flinching responses during the time course of the formalin test, resulting in a state of attenuated hyperalgesia on day 35.

*Effect of pharmacological intervention on lipid peroxidation*

As shown in Table 2, diabetic animals had a significantly increased level of thiobarbituric acid reactive substance (TBARS) in sciatic nerve after 5 weeks of STZ injection diabetes, as compared to age-matched normal control animals.

Monotherapy with any of the drug for 3 weeks failed to attenuate the sciatic nerve TBARS level in diabetic rats. However, a high dose of thalidomide (50 mg $kg^{-1}$) and subeffective dose combination of (dipyrone 300 mg $kg^{-1}$ and thalidomide 25 mg $kg^{-1}$) significantly attenuated oxidative stress as manifested by decreased TBARS level. In addition, pretreatment with thalidomide (50 mg $kg^{-1}$) was also able to reduce the level

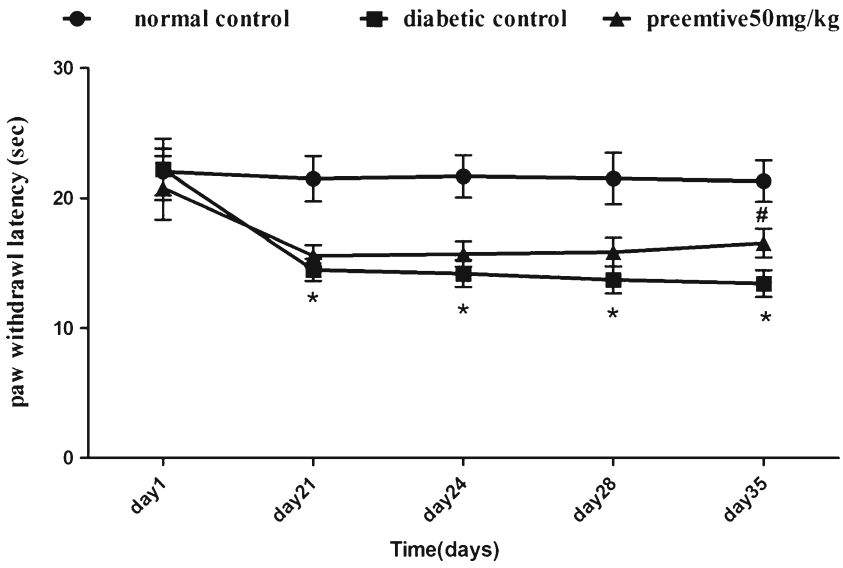



**Fig. 2** Effect of pretreatment with thalidomide 50 mg $kg^{-1}$, starting 1 day before diabetes induction, on development of thermal hyperalgesia in diabetic rats. Values are expressed as mean ± SD. $n=6$. *$p<0.05$ vs. normal control; $^{\#}p<0.05$ vs. diabetic control. Preemptive treatment with thalidomide (50 mg/kg) in diabetic rats

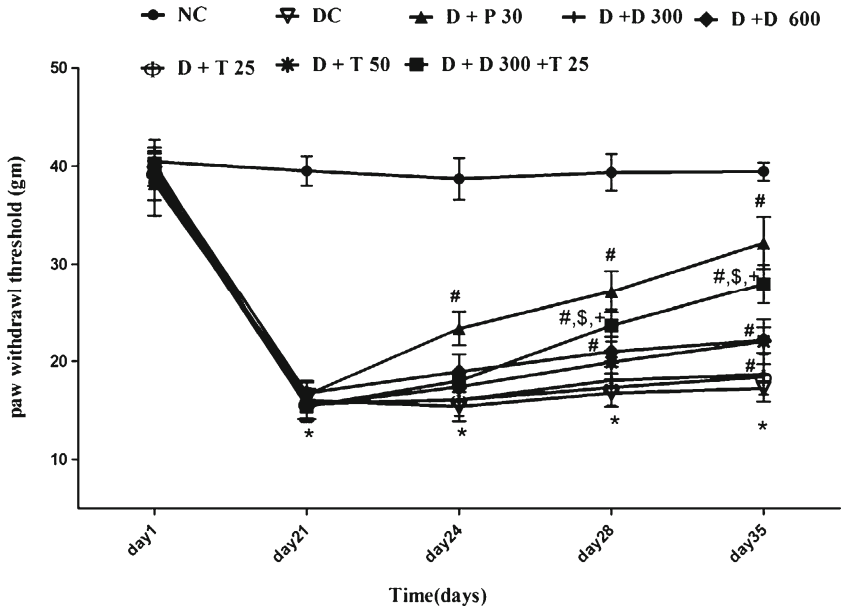



**Fig. 3** Effect of dipyrone and thalidomide alone or in combination on mechanical allodynia, in control and STZ injected diabetic rats. Values are expressed as mean±SD. $n=6$. *$p<0.05$ vs. normal control; $^{\#}p<0.05$ vs. diabetic control; $^{\$}p<0.05$ vs. dipyrone (300 mg/kg); $^{+}p<0.05$ vs. thalidomide (25 mg/kg). *NC* normal control; *DC* diabetic control; *D + P 30* pregabalin (30 mg kg$^{-1}$)-treated group; *D + D 300 and D + D 600* dipyrone (300 and 600 mg kg$^{-1}$)-treated group; *D + T 25 and D + T 50* thalidomide (25 and 50 mg kg$^{-1}$)-treated group; *D + D300 + T 25* dipyrone (300 mg kg$^{-1}$)-and thalidomide (25 mg kg$^{-1}$)-treated combination group. *Arrow* indicates day of initiation of treatment

of TBARS significantly. Three-week treatment with pregabalin (30 mg kg$^{-1}$) in diabetic animals produced a significant reduction in TBARS levels in sciatic nerve.

### *Effect of pharmacological intervention on reduced glutathione*

As shown in Table 2, 5-week treatment of diabetic animals showed a significantly decreased level of GSH in sciatic nerve, as compared to age-matched control animals. Pregabalin (30 mg kg$^{-1}$)-treated rats in the positive control group showed improved GSH level of sciatic nerve of diabetic rats on day 35, as compared to diabetic untreated rats, whereas monotherapy with low and high doses of dipyrone (300 and 600 mg kg$^{-1}$) and thalidomide (25 and 50 mg kg$^{-1}$) and pretreatment with thalidomide (50 mg kg$^{-1}$) did not improve the reduced GSH level in diabetic rats. On the other hand, subeffective dose low combination of dipyrone 300 mg kg$^{-1}$ and thalidomide 25 mg kg$^{-1}$ showed significant improvement in GSH level in sciatic nerve of diabetic rats compared to untreated diabetic rats.

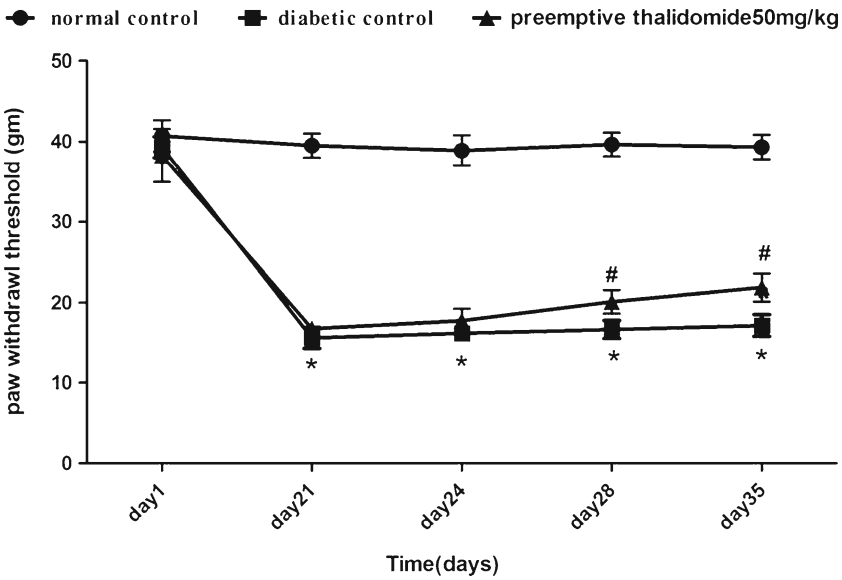



**Fig. 4** Effect of pretreatment with thalidomide 50 mg kg$^{-1}$, starting 1 day before diabetes induction, on the development of mechanical allodynia in diabetic rats. Values are expressed as mean±SD. $n=6$. *$p<0.05$ vs. normal control; $^{\#}p<0.05$ vs. diabetic control. Pretreatment with thalidomide in diabetic rats (50 mg/kg)

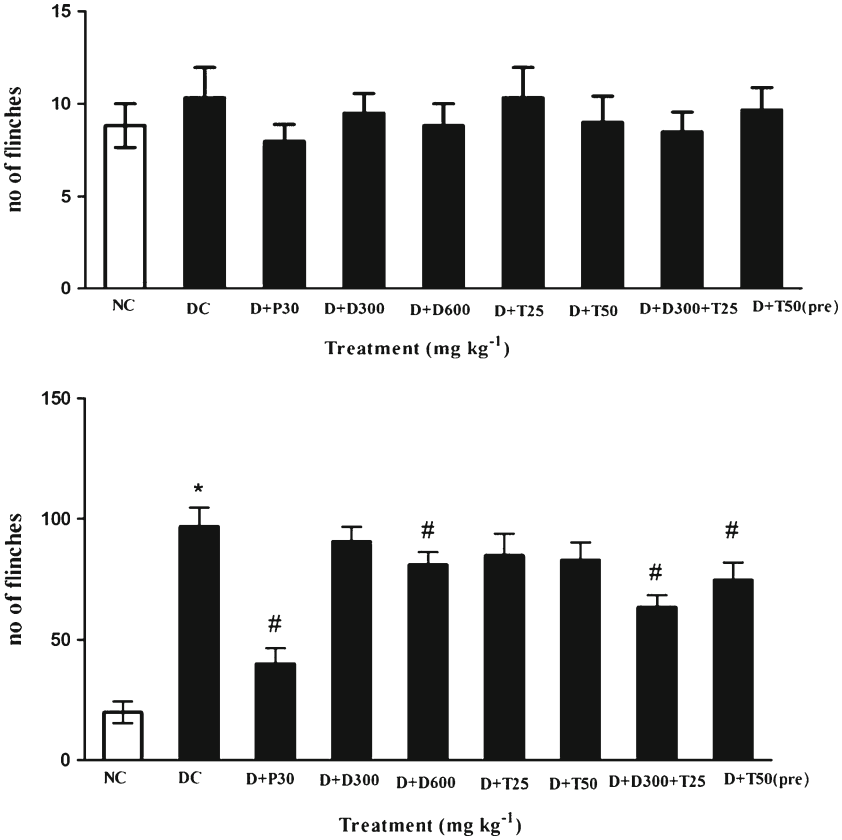



**Fig. 5** Effect of dipyrone and thalidomide alone or in combination on phase 1 and 2 nociceptive responses in the formalin test indicative of hyperalgesia on the development of diabetic neuropathy. Values are expressed as mean±SD. $n$=6. *$p$<0.05; #$p$<0.05 vs. diabetic control. *NC* normal control; *DC* diabetic control; *D + P 30* pregabalin (30 mg kg$^{-1}$)-treated group; *D + D 300 and D + D 600* dipyrone (300 and 600 mg kg$^{-1}$)-treated diabetic group; *D + T 25 and D + T 50* thalidomide (25 and 50 mg kg$^{-1}$)-treated diabetic group; *D + D 300 + T 25* dipyrone (300 mg kg$^{-1}$)- and thalidomide (25 mg kg$^{-1}$)-treated combination group

*Effect of pharmacological intervention on nitrite level*

As shown in Table 2, serum NO levels were significantly elevated in the diabetic rats. Treatment with dipyrone (300 mg kg$^{-1}$) and thalidomide (25 and 50 mg kg$^{-1}$) had no significant effect on the serum nitrite level up to 2 weeks. Conversely, administration of dipyrone (600 mg kg$^{-1}$) and subeffective dose combination of dipyrone 300 mg kg$^{-1}$ and thalidomide 25 mg kg$^{-1}$ significantly reduced the elevated level of nitrite in diabetic rats compared to untreated diabetic control rats on day 35. Similarly, pretreatment with thalidomide (50 mg kg$^{-1}$) was also able to attenuate the elevated serum nitrite level in diabetic rats compared with age-matched untreated diabetic rats. Pregabalin (30 mg kg$^{-1}$)

**Table 2** Effect of dipyrone and thalidomide alone or in combination on (sciatic nerve) TBARS, GSH level, and (serum) nitrite level in rats

| Treatment (mg kg$^{-1}$) | TBARS (μmol/g tissue) | GSH (μmol/g tissue) | Nitrite (μmol/l) |
|---|---|---|---|
| NC | 0.15±0.051 | 7.1±0.586 | 33±8.717 |
| DC | 0.48±0.056* | 3.45±0.427* | 98±2.280* |
| D + P 30 | 0.22±0.042 # | 4.73±0.225# | 54.33±4.501# |
| D + DPN 300 | 0.40±0.046 | 3.85±0.450 | 90±5.76 |
| D + DPN 600 | 0.36±0.053# | 4.3±0.670 | 75±2.607# |
| D + TH 25 | 0.39±0.045 | 3.68±0.213 | 84±2.68 |
| D + TH 50 | 0.36±0.053 | 4.13±0.382 | 79±2.96 |
| D + DPN 300 + TH 25 | 0.29±0.039# | 4.6±0.328# | 71±1.897# |
| D + TH 50 (pre) | 0.388±0.027# | 4.08±0.196 | 81.16±2.71# |

Values are mean±SD

*NC* normal control; *DC* diabetic control; *D + P 30* pregabalin-treated diabetic rats; *D + DPN 300 and D + DPN 600* dipyrone-treated diabetic rats; *D + TH 25 and D + TH 50* thalidomide-treated diabetic rats; *D + DPN 300 + TH 25* dipyrone and thalidomide combination-treated diabetic rats; *D + TH 50 (pre)* pretreatment with thalidomide in diabetic rats; *GSH* reduced glutathione

*$p$<0.05 vs. normal control

# $p$<0.0.5 vs. diabetic control

treatment for 2 weeks significantly attenuated an increase in NO levels.

## Discussion

This study demonstrated the effect of dipyrone, a central and peripheral COX inhibitor, and thalidomide, a TNF-α inhibitor, on the development and maintenance of STZ-induced pain behavior in rats.

Studies in the experimental animal models such as the STZ-induced diabetic model had helped to define the pathophysiology of diabetic neuropathic pain. It is a well-established fact that diabetic rats display exaggerated hyperalgesic behavior in response to noxious stimuli that may mimic the aspects of painful diabetic neuropathy in humans (Freshwater et al. 2002), and for this reason, STZ-diabetic rats have been increasingly used as a model of painful diabetic neuropathy.

It has been reported earlier that STZ-induced diabetic neuropathic pain is characterized by hyperalgesia and allodynia (Meeus and Nijs 2007; Velazques et al. 2007) and was also found in the present study after the third week following STZ injection. This is in line with various other observations (Kuhad et al. 2008; Ohsawa and Kamei 1999). However, some studies suggest that STZ-induced hypernociception is not only associated with hyperglycemia (Romanovsky et al. 2004); there is a possibility of STZ sensitizing the peripheral afferent nociceptors and central nociceptive neurons (Cunha et al. 2009). Furthermore, diabetic rats showed an increased frequency of flinching following paw formalin injection that is indicative of hyperalgesia in this model (Courteix et al. 1993). In this study, diabetic rats displayed exaggerated flinching behavior only in the second phase of the formalin test in diabetic animals, which is in agreement with the other report (Tourandokht et al. 2005).

The pathogenesis of NP is complex and yet to be explored. It is well documented that oxidative stress in diabetes plays a key role in modulating diabetes-induced thermal hyperalgesia and mechanical allodynia, thereby altering the pain perception (Shukla and Tang Wang 2006). Hyperglycemia is reported to induce oxidative stress through multiple pathways such as redox imbalances secondary to enhanced aldose reductase activity (Yagihashi et al. 2001); increased advanced glycation end-products (Brownlee et al. 1988); altered protein kinase C activity, especially b-isoforms (Cameron et al. 1999); prostanoid imbalances (Pop-Busui et al. 2002); and mitochondrial overproduction of superoxide (Brownlee 2003). All these pathways converge in the production of oxidative stress. Key mediators of oxidative stress in the progression and development of diabetic neuropathy are marked increase in ROS, higher concentration of nitrite (an index of amount of NO released, which is a source of peroxynitrite), and the decreased antioxidant defenses in the tissue of diabetic animals (Schmeichel et al. 2003). Oxidative stress has been documented in peripheral nerve (Cameron et al. 1999; Obrosova et al. 1998; Song et al. 2003), dorsal root and sympathetic ganglia (Low et al. 1997), and the vasculature of the peripheral nervous system (Coppey et al. 2001) and contributes to nerve blood flow and conduction deficits, impaired neurotrophic support, changes in signal transduction and metabolism, and morphological abnormalities characteristic of PDN (Pop-Busui et al. 2006a, b). In the present study, there was a significant increase in the various markers of oxidative stress such as TBARS, nitrite, and reduction in endogenous antioxidant enzymes activity, that is, reduced glutathione in STZ-treated rats compared with vehicle-treated control rats. The STZ-injected rats had significantly higher blood glucose level and decreased body weight that was observed throughout study.

Furthermore, increased oxidative stress triggers NF-κB (Faux and Howden 1997), which consequently leads to TNF-α activation (Ignatowski et al. 1999; Kuhad et al. 2008), COX-2 mRNA induction (Kiritoshi et al. 2003), and COX-2 gene expression (Pop-Busui et al. 2006a, b). COX-2 up-regulation increases the rate of prostaglandin G2 (PGG2) to PGH2 conversion and ROS generation, further exacerbating oxidative stress. COX inhibitors have been reported to ameliorate pain behavior in rats. COX inhibitors such as indomethacin and piroxicam have been reported to prevent the neuropathic pain behavior in experimental model. However, dipyrone, COX inhibitor, at a low dose that was used in this study, on the basis of pilot study in rats ($n$=4), failed to provide a beneficial effect, but a higher dose significantly attenuated the STZ-induced hyperalgesia and allodynia.

Another inflammatory enzyme regulated by NF-κB is inducible NO synthase (iNOS) (Kim et al. 2008). Like COX-2, iNOS both induces and is induced by NF-κB, leading to a vicious cycle of inflammation (Kim et al. 2008). The NO generated by iNOS directly modulates the blood supply to the nerves and participates in microvascular changes following injury (Levy and Zochodne 2004). Excessive local levels of NO during inflammation may damage axons and growth cones. NO avidly combines with superoxide to form peroxynitrite, which rapidly causes protein nitration or nitrosylation, lipid peroxidation, DNA damage, and cell death, and has direct toxic effects on the nerve tissue leading to neuropathic pain (Kim et al. 2008). Although the level of peroxynitrite was not measured, NO, an indicator of nitrosative stress, was measured and found to be increased in the STZ-diabetic rats.

Under chronic hyperglycemia, oxidative stress accelerates endogenous TNF-α production in microvascular and neural tissues, which undergo an increased microvascular permeability, hypercoagulability, and nerve damage, thus initiating and promoting the development of characteristic lesions of diabetic microangiopathy and polyneuropathy (Satoh et al. 2003). Furthermore, TNF-α up-regulates COX-2 enzyme, resulting to an

enhanced level of PGs (Campbell and Meyer 2006; Yi et al. 2007). In one study, administration of TNF-$\alpha$ significantly decreased motor nerve conduction velocity (MNCV) in diabetic rats, although it did not influence the MNCV in nondiabetic rats (Satoh et al. 1998). This finding implies that TNF-$\alpha$ contributes to diabetic nerve dysfunction and indicates that suppression of enhanced TNF-$\alpha$ production in a diabetic state might attenuate the progression of diabetic polyneuropathy. Moreover, TNF-$\alpha$ has been reported to initiate the release of other inflammatory cytokines including IL-1$\beta$ and IL-2 that are responsible for causing neuropathic pain (Watkins and Maier 2003; Wang et al. 2006). In addition, proinflammatory cytokines release leads to accumulation of free radicals (Leite et al. 2007) and activates enzymes like COX-2 and iNOS, further releasing PGs and NO, well-known mediators that are involved in spinal hypersensitization (Thacker et al. 2007). Therefore, it seems that TNF-$\alpha$ production is involved in the incipient stage of diabetic peripheral neuropathy.

In the present study, monotherapy with a high dose of dipyrone (600 mg kg$^{-1}$, i.p.) and thalidomide (50 mg kg$^{-1}$) partially corrected the altered thermal hyperalgesia and mechanical allodynia in diabetic animals. However, subeffective dose combination of dipyrone (300 mg kg$^{-1}$, i.p.) and thalidomide (25 mg kg$^{-1}$, i.p.) reversed STZ-induced thermal hyperalgesia and allodynia. Moreover, preemptive treatment with thalidomide prevented the development of STZ-induced thermal hyperalgesia and mechanical allodynia. Our results are in full agreement with Zanella et al. (2008) and Dogrul et al. (2011), who reported on improvement in thermal hyperalgesia with etanercept (TNF-$\alpha$-antibodies). In contrast, recently, it has been reported that TNF-$\alpha$ elevates neurite outgrowth through an NF-$\kappa$B-dependent pathway in cultured adult sensory neurons, and the diminished expression of TNF-$\alpha$ in diabetes may contribute to sensory neuropathy (Saleh et al. 2011).

In addition, formalin-induced flinching response in phase 2 was inhibited by dipyrone (600 mg kg$^{-1}$, i.p.)-treated diabetic group and the subeffective dose combination of dipyrone (300 mg kg$^{-1}$, i.p.) and thalidomide (25 mg kg$^{-1}$, i.p.). Also, in thalidomide (50 mg kg$^{-1}$, i.p.) preemptive treatment group, the exaggerated flinching behavior was blunted. Thus, it is clear from the behavioral studies that preemptive thalidomide and subeffective dose combination of dipyrone and thalidomide attenuated the development of dipyrone. In the present study, we have targeted oxidative stress and antioxidant defence factors in diabetic neuropathy by the inhibition of TNF-$\alpha$ (proinflammatory cytokine) and COX inhibition. Monotherapy with high-dose dipyrone (600 mg kg$^{-1}$, i.p.), subeffective dose combination of dipyrone (300 mg kg$^{-1}$, i.p.) and thalidomide (25 mg kg$^{-1}$, i.p.), and preemptive thalidomide (50 mg kg$^{-1}$, i.p.) resulted in the reduction of oxidative stress, particularly TBARS in sciatic nerve of diabetic rats; whereas only the combination of dipyrone- and thalidomide-treated group restores the endogenous antioxidant GSH in sciatic nerve of diabetic rats. Moreover, the combination therapy and preemptive thalidomide treatment reduced the serum nitrite level in diabetic rats, thereby reducing nitrosative stress.

Hence, it may be concluded that subeffective dose combination of thalidomide and dipyrone significantly inhibited STZ-induced neuropathic pain behaviors.

## Conclusion

The results of the present study demonstrate that the combination of subeffective dose of dipyrone and thalidomide prevents the development and maintenance of experimental diabetic neuropathy and that their antihyperalgesic and antiallodynic effects are mediated by inhibition of TNF-$\alpha$ and COX activation and by modulating oxidative and nitrosative stress in sciatic nerve.

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