European Journal of Pharmacology 601 (2008) 79–87

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and Analgesia

Minocycline prevents the development of neuropathic pain, but not acute pain: Possible anti-inflammatory and antioxidant mechanisms

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ARTICLE INFO

Article history:
Received 21 April 2008
Received in revised form 28 September 2008
Accepted 10 October 2008
Available online 18 October 2008

Keywords:
Allodynia
Glia
Hyperalgesia
Hypersensitivity
Microglia
Minocycline
Nerve injury
Neuropathic pain

ABSTRACT

Glia, particularly astrocytes and microglia, are known to play an important role in central sensitization and are strongly implicated in the exaggerated pain states. In the present study, we determined the effect of minocycline, an inhibitor of microglial activation, in acute nociception, peritonitis, and the development and maintenance of hypersensitivity following chronic constriction injury of the sciatic nerve in rats. A single dose of minocycline (30 or 100 mg/kg, i.p.) 30 min before acetic acid or zymosan injection did not attenuate the nociceptive behavior in mice. It had no effect on the early events of peritoneal inflammation (vascular permeability, inflammatory cell infiltration, and release of pro-inflammatory cytokines) in acetic acid or zymosan-injected mice. In addition, minocycline (30 or 100 mg/kg, i.p.) did not alter basal nociceptive responses in the tail immersion test. Chronic administration of minocycline (10 or 30 mg/kg, i.p.) for 7 days started before nerve injury significantly prevented the development of neuropathic pain, interestingly, it further delayed the development of hypersensitivity. In contrast, single injection of minocycline failed to reverse hypersensitivity when administered during the development of neuropathic pain. No significant effects were observed on hypersensitivity when treatment was started once neuropathic state was established. Pre-treatment, but not post-treatment, with minocycline markedly attenuated increased pro-inflammatory cytokines release and oxidative and nitrosative stress in mononeuropathic rats. These results suggest that minocycline had no effect on acute peritoneal inflammation, nociception, and chronic administration of minocycline when started early before peripheral nerve injury could attenuate and further delays the development of neuropathic pain. Concluding, this study clearly shows minocycline, an inhibitor of microglial activation, by inhibiting the release of pro-inflammatory mediators and reducing oxidative stress prevented the development of neuropathic pain.

1. Introduction

Peripheral nerve injury and inflammation often induce exaggerated pain states characterized by sensitization of peripheral and central primary afferent neurons. It has been proposed that inflammatory reactions in the injured nerves contribute to the generation and maintenance of neuropathic pain (Ma and Eisenach, 2003; Ma and Quirion, 2005). When acetic acid or zymosan injected intraperitoneally or a peripheral nerve is injured, there is recruitment of inflammatory cells from the circulation, and over production of cytokines or mediators which activate Aδ and C fibers in sensory nerves producing pain, hyperalgesia, or allodynia in both humans and experimental animals (Ma and Quirion, 2005; Ribeiro et al., 2000) Recently, investigators have placed emphasis on the role of immune cells, such as invading macrophages and non-neuronal cells of the spinal cord, in nociceptive processing and the exaggerated pain states. Growing body of evidence indicates that the glial cells, particularly microglia (CNS macrophages) and astrocytes are activated following peripheral and central noxious insult and their activation is thought to play an important role in central sensitization (Owolabi and Saab, 2006; Zhuang et al., 2005, 2006). It is well known that activated glia increases the release of various pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α (Kreutzberg, 1996; Watkins et al., 2001). Further, both the glia and neurons express receptors for various neurotransmitters and neuromodulators involved in central sensitization (Clark et al., 2007; Watkins et al., 2001). The recognition of glia as powerful modulator of nociception stimulated the search for agents that specifically inhibit the activation and metabolism of glial cells leading to the discovery of glial modulators which showed antiallodynic and antihyperalgesic properties in various models of experimental pain (Ledeboer et al., 2007; Mika et al., 2007).

Minocycline is a semisynthetic second generation tetracycline that exerts anti-inflammatory effect that is completely separate and distinct from its antimicrobial action (Tikka et al., 2001). It is a lipophilic molecule absorbed rapidly and readily cross the blood-brain barrier (Aronson,

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doi:10.1016/j.ejphar.2008.10.018

1980). It selectively disrupts the activation of microglia without directly affecting neurons or astroglia (Raghavendra et al., 2003; Tikka et al., 2001). Inhibition of microglial activation has also been demonstrated *in vitro* (Yenari et al., 2006) and in experimental models of acute and chronic brain insults (Chen et al., 2000; Tikka et al., 2001; Wu et al., 2002). In the brain, it showed neuroprotection by inhibiting inflammation, decreased free radical formation by inhibiting inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and inhibits the caspase-1 in experimental model of Parkinson's and Huntington's diseases, and prevented *N*-methyl-D-aspartate mediated neurotoxicity (Liu and Hong, 2003; Tikka and Koistinaho, 2001). Recently, its antihyperalgesic and antiallodynic effects have been demonstrated in models of arthritis, spinal nerve transection, sciatic inflammatory neuritis (Ledeboer et al., 2005; Raghavendra et al., 2003; Shan et al., 2007).

To date, there are no studies that evaluated the effects of minocycline on tail immersion test that provides information on spinal sensory processing and also inflammatory events that precede acute nociception in the writhing tests. In addition, studies on its pre-treatment and post-treatment effects in attenuating and/or reversing hypersensitivity, respectively, following chronic constriction injury may be beneficial in understanding the effect of minocycline on ongoing pain related behaviors. Thus, there are not only mechanistic reasons to examine the time-dependent effect of minocycline, an inhibitor of microglial activation, but also practical approaches to evaluate long lasting effects in attenuating hypersensitivity in mononeuropathic rats. In the present study, we examined the effects of minocycline on tail flick response and early inflammatory events (vascular permeability, infiltration of inflammatory cells, and release of pro-inflammatory cytokines) leading to nociception in mice. We also investigated the effect of acute as well as chronic systemic administration of minocycline on development and maintenance of hypersensitivity following chronic constriction injury of the sciatic nerve in rats. Further, its effect on the release of pro-inflammatory cytokines and oxidative stress was also evaluated to elucidate mechanism of action of minocycline in attenuating neuropathic pain. Part of this study has been previously published in abstract form (Padi and Kulkarni, 2005).

2. Materials and methods

2.1. Experimental animals

Male Swiss mice (20–26 g) and Wistar rats (150–180 g) (Central Animal House of Panacea Biotec Ltd., India) were used in the experiments. All procedures involving the use of animals were approved by the Institutional Animal Ethics Committee and carried out in accordance with the guidelines of the Indian National Science Academy. All the animals and the anesthetized rats following surgery were kept under standard conditions of light and dark cycle with food and water *ad libitum* in groups of 3 animals in plastic cages with soft bedding.

2.2. Drugs and drug administration

Evans blue and zymosan A (a cell wall component of yeast *Saccharomyces cerevisae*, Sigma-Aldrich, India), minocycline hydrochloride (Wyeth-Lederle Pharmaceuticals, India), and glacial acetic acid (SD Fine Chemicals, India) were used in this study. All other chemicals were of the highest commercial grade available. The drug solutions for intraperitoneal (i.p.) administration were freshly prepared by suspending them in one or two drops of Tween 80 in normal saline and administered 1 ml/100 g rat and 0.1 ml/10 g mouse. Glacial acetic acid and zymosan were diluted and Evans blue was suspended in sterile normal saline.

2.3. Behavioral test paradigm

2.3.1. Writhing test

Nociceptive activity was tested in mice using the writhing models of visceral pain as described in previous studies (Padi and Kulkarni, 2004; Vale et al., 2003). Briefly, mice were allowed to acclimate in Plexiglas chambers for 30 min before injection of nociceptive stimuli. Animals were gently restrained and the nociceptive stimuli were injected into the peritoneal cavities of mice and the intensity of nociception was quantified by counting the total number of writhes for 20 min, starting 3 min after the administration of the acetic acid solution and between 0 and 30 min after zymosan injection. A writhe was defined as contraction of the abdominal muscles accompanied by elongation of the body and hind limbs. The doses of the nociceptive stimuli were: zymosan (1 mg per mouse; 0.5 ml of 2 mg/ml solution), acetic acid (0.1 ml per 10 g body weight of a 1% solution, v/v).

2.3.2. Tail immersion (warm water) test

Tail of mice was immersed in a water bath maintained at 52±0.5 °C until tail withdrawal (flicking response) or signs of struggle were observed. The baseline latency of tail withdrawal from thermal source was established three times, 5 min apart and averaged. A cut-off time of 15 s was imposed to avoid injury to the tail. Nociceptive latency was measured at 0 (baseline), 15, 30, 60, 90, 120 and 180 min after minocycline administration and expressed as mean latency. The nociceptive response in the tail immersion test reflects activity of a simple spinal reflex and is generally attributed to spinal sensory processing (Ibrahim et al., 2006).

2.3.3. Chronic constriction nerve injury

The unilateral mononeuropathy was produced according to the method described by Bennett and Xie (1988). Briefly, the rats were anesthetized using 40 mg/kg sodium pentobarbital intraperitonelly (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. Proximal to the sciatic trifurcation, approxmately 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.

2.3.3.1. Assessment of neuropathic pain. Allodynia (heightened response to normally non-noxious stimuli) and hyperalgesia (decreased threshold to noxious stimuli) were evaluated in both sham and chronic sciatic nerve constriction injured (CCI) rats.

2.3.3.1.1. Cold allodynia. Cold sensitivity was quantified by measuring the duration of paw withdrawal in response to acetone application. An acetone drop was formed at the end of a piece of a small polyethylene tube, which was connected with a syringe. The drop was gently applied to the plantar surface of the hind paws. The duration of time that the animal spent lifting, shaking, or licking the acetone applied hind paw was recorded during a 2 min period that started immediately after acetone application.

2.3.3.1.2. Mechanical hyperalgesia. Mechanical nociceptive thresholds were evaluated using Randall-Selitto apparatus (Ugo Basile, Italy) by applying noxious pressure to hind paw. In brief, by pressing a pedal that activated a motor, the force is increased at a constant rate on a linear scale. When the animal displayed pain by withdrawal of the paw, the pedal was immediately released, and the nociceptive pain threshold was read on the scale. The paw withdrawal threshold was expressed in grams and a cut-off threshold of 500 g was used to avoid potential tissue injury (Padi et al., 2004a).

2.3.4. Induction of peritonitis

Acetic acid or zymosan-induced peritoneal inflammation was induced as according to Doherty et al. (1995). A freshly prepared acetic acid (0.1 ml per 10 g body weight of a 1% solution, v/v) or zymosan A (0.5 ml per animal of 2 mg/ml solution) was injected intraperitoneally and each mouse was sacrificed by cervical dislocation

30 min after inflammatory stimuli. The peritoneal cavity was lavaged with 1.5 ml of saline, and after a 30 s gentle manual massage, total exudate was retrieved. The amount of exudate was calculated by subtracting the volume injected (1.5 ml) from the total volume recovered (Δ of volume increase in ml).

2.3.5. Vascular permeability

Evans blue dye (10 mg/ml in saline, 0.2 ml/mouse) was injected intravenously just before the injection of zymosan. Thirty minutes after intravenous injection, animals were killed and the peritoneal cavity lavaged as described in Section 2.3.4, "Induction of peritonitis". The lavage fluid was centrifuged at 3000 ×*g* for 3 min and the absorbance of Evans blue in the supernatant measured at 650 nm. Evans blue binds to plasma albumin and distributes with albumin throughout the animal. Therefore, the accumulation of Evans blue dye in the peritoneal cavity is a quantitative index of increased vascular permeability and extravasation of albumin (Doherty et al., 1995).

2.3.6. Cell counts

Total and differential cell counts were done by injecting a 200 µl of mouse peritoneal exudate obtained following saline, acetic acid or zymosan injection into Swelab Cell Counter 920^{EO}.

2.4. Pro-inflammatory cytokines

The peritoneal exudate collected 30 min after vehicle or zymosan injection was centrifuged at 3000 ×*g* for 3 min. The supernatant was used for the estimation of TNF-α and the levels are expressed as pg/cavity. The levels of IL-1β and TNF-α were also quantified in sciatic nerves of rats. The injured sciatic nerves were isolated on day 15 (pre-treatment groups) or 29 (post-treatment groups) post-surgery and weighed sections were homogenized in homogenization buffer. The samples were cold centrifuged and the supernatant was used for estimation of IL-1β and TNF-α protein concentrations 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 and are expressed as pg/mg protein.

2.5. Markers of oxidative stress

Each rat was sacrificed by cervical dislocation and the two sciatic nerves of each rat were removed by giving a blunt cut in the thigh.

2.5.1. Lipid peroxidation

Lipid peroxidation in sciatic nerve was estimated colorimetrically by measuring thiobarbituric acid reactive substances by the method of Niehaus and Samuelson (1968). In brief, 0.1 ml of supernatant of 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 water bath for 15 min, cooled and centrifuged and then clear supernatant was measured at 535 nm against blank. The values are expressed as µmol/g wet weight.

2.5.2. Reduced glutathione

Reduced glutathione (GSH) levels in sciatic nerve were determined according to Lou et al. (1988). In brief, 100 µl of supernatant was mixed with 0.89 ml of 1.0 M Tris, pH 8.2, and 0.02 M EDTA. A 10 µl of dithionitrobenzene was added and the absorbance was measured at 412 nm. The results are expressed as µg/mg wet weight.

2.5.3. Total nitrite/nitrate

The sciatic nerve total nitrite and nitrate was estimated according to the procedure described by Sastry et al. (2001). In brief, 400 µl of carbonate buffer, pH 9.0, was added to 100 µl of nerve supernatant followed by a small amount (0.15 g) of copper-cadmium filings and then incubated at room temperature for 1 h with thorough shaking. The reaction was stopped by the addition of 100 µl of 0.35 M sodium hydroxide followed by 400 µl of 120 mM zinc sulfate solution under vortex, the solution was allowed to stand for 10 min, and centrifuged at 4000 ×*g* for 10 min. To a 500 µl of the clear supernatant 250 µl of 1.0% sulfanilamide and 250 µl of 0.1% N-naphthylethylenediamine were added with shaking. After 10 min the absorbance was measured at 545 nm against a blank. The concentration of nitrite in the supernatant are expressed as µmol/g wet weight.

2.6. Study design

All animals were acclimatized to laboratory environment for at least 2 h before testing. The paw withdrawal responses to thermal and mechanical stimulation were measured on day 0 before performing surgery in rats. To investigate whether minocycline produce any effect on acute nociception, rats were administered intraperitoneally (i.p.) with vehicle or minocycline (30 or 100 mg/kg,) 30 min before acetic acid or zymosan injection and immediately observed for nociceptive behavior. In the same set of animals, peritoneal exudate was also collected and measured the change in exudate volume, total and differential cell counts, pro-inflammatory cytokines 30 min after acute nociceptive stimuli to investigate whether minocycline is able to prevent the development of acute peritoneal inflammation and nociception. A separate set of animals were administered with zymosan to measure vascular permeability. To investigate whether minocycline, an inhibitor of microglial actvation, is able to prevent the development of hyperalgesia following nerve injury, a set of rats were pre-treated with vehicle or minocycline (10 or 30 mg/kg, i.p.) administered 2 h before surgery and continued once daily for 7 days (pre-treatment) following nerve injury. The paw withdrawal response to thermal and mechanical stimulation was tested on day 0 (baseline), 4, 7, 10, 14, and once a week thereafter for 4 weeks after nerve injury. One subset of animals (n=6–8) was sacrificed on day 15 to measure the markers of oxidative stress and pro-inflammatory cytokines and the other subset of animals (n=6–8) were evaluated for long-term effects of minocycline without treatment for another two weeks. To investigate whether single dose of minocycline affects the development of hypersensitivity, minocycline was administered during the development (on day 4 post-surgery). A single dose of vehicle or minocycline (10 or 30 mg/kg, i.p.) was administered to CCI rats on day 4 and the paw withdrawal responses to cold and mechanical stimulation was tested 2 h before minocycline administration, 0.5, 1, 2, 4, and 24 h after treatment. Further, to determine the effect of minocycline on the maintenance of hypersensitivity, vehicle or minocycline (10 or 30 mg/kg, i.p.) were administered to separate group of rats after behavioral assessment on day 7 following nerve injury and continued once daily for 7 days (post-treatment). Since, administration of minocycline for one week failed to reverse established neuropathic pain, the treatment was continued for another 14 days i.e. till day 28. The paw withdrawal response to thermal and mechanical stimulation was tested on day 0, 7 (2 h before minocycline administration), 10, 14, 17, 21, and 28 after nerve injury. The behavioral tests were started 2 h after the injection at days mentioned in chronic vehicle or minocycline treated groups. All the post-treated animals were sacrificed on day 29 to measure the markers of oxidative stress and pro-inflammatory cytokines.

2.7. Statistics

All the values are expressed as mean ± S.E.M. for at least six animals per group. The behavioral data was analyzed by one-way repeated measures analysis of variance (RM-ANOVA) whereas the results of the markers of inflammation and oxidative stress were analyzed by

one-way ANOVA (SigmaStat Version 2.0, SPSS Inc., Chicago, IL, USA). In both the cases Tukey's test was used for multiple comparisons. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of minocycline on acetic acid or zymosan-induced nociception in mice

In mice administered with vehicle, subsequent injection of either acetic acid or zymosan into peritoneal cavity resulted in visceral nociceptive responses characterized by robust abdominal constrictions and extension of hind limbs. Administration of minocycline (30 or 100 mg/kg, i.p.), 30 min before injection of nociceptive stimuli, had no effect on writhing response as compared to vehicle-treated mice (Fig. 1A and B).

3.2. Effect of minocycline on vascular permeability in zymosan-injected mice

The absorbance due to Evans blue, which is nonpermeable generally, was increased in the supernatant of the peritoneal exudate following intraperitoneal administration of zymosan as compared to saline-treated mice indicating increased vascular permeability, a first step in inflammation responsible for infiltration of inflammatory cells. Treatment with minocycline (30 or 100 mg/kg, i.p.) 30 min prior to zymosan did not alter increased absorbance as compared to control mice (Fig. 2).

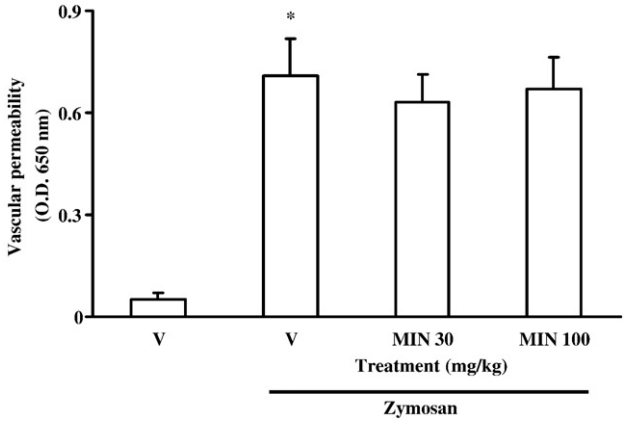

Fig. 2. Effect of minocycline on vascular permeability in zymosan-injected mice. Animals were either vehicle treated (V) or received minocycline (MIN; 30 or 100 mg/kg, i.p.) 30 min before i.p. injection of zymosan (1 mg in 0.5 ml) followed by i.v. injection of Evans blue. The amount of blue dye was estimated at 650 nm in peritoneal exudate retrieved 30 min after zymosan/dye injection. Pre-treatment with minocycline had no effect on zymosan-induced vascular permeability. All results are shown as mean ± S.E.M in groups of 6–8 mice. *$P<0.05$ vs saline control group.

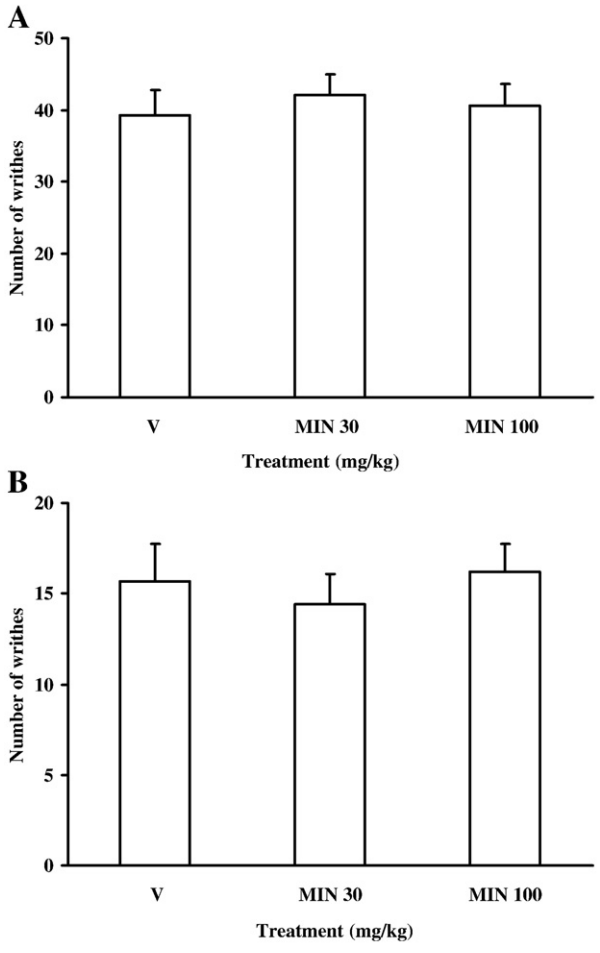

Fig. 1. Effect of minocycline treatment on acetic acid or zymosan-induced nociception in mice. The bars show the number of writhes induced by intraperitoneal administration of (A) a solution of acetic acid (1% v/v; 0.1 ml/10 g) and (B) zymosan (2 mg/ml; 1 mg/cavity) after administration with vehicle or minocycline (MIN; 30 or 100 mg/kg, i.p.). Minocycline was intraperitoneally administered 30 min before nociceptive assay. Minocycline had no effect on nociceptive stimuli-induced writhes in mice. The data represent the mean ± S.E.M. of writhing responses during 20 or 30 min intervals after injection of a solution of acetic acid or zymosan, respectively. (n = 6–8 in each group).

3.3. Effect of minocycline on exudate volume, total and differential cell counts in the peritoneal exudate of acetic acid or zymosan-injected mice

In mice injected with either acetic acid or zymosan into peritoneal cavity, the mean change in exudate volume 30 min after stimuli were significantly different from that of saline-injected animals. Among the polymorphonulear cells and mononuclear cells invaded neutrophils and macrophages, respectively, were the majority of the inflammatory cells following peritoneal inflammation. Pre-treatment with a single dose of minocycline (30 or 100 mg/kg, i.p.) had no effect on peritoneal exudate, total cells, and differential cell count following acetic acid or zymosan injection (Table 1).

3.4. Effect of minocycline on TNF-α level in the peritoneal exudate of acetic acid or zymosan-injected mice

There was a significant increase in TNF-α level in the peritoneal exudate following acetic acid or zymosan injection. However, pre-treatment with minocycline had no effect on the peritoneal exudate levels of TNF-α in either acetic acid or zymosan-injected mice (Table 1).

3.5. Effect of minocycline on tail immersion test in mice

The mean baseline tail withdrawal latency to thermal (52 °C) stimulation in the tail immersion test obtained for each mouse was 3.55 ± 0.71 s. Systemic administration of minocycline (30 or 100 mg/kg, i.p.) had no effect on tail withdrawal latency as compared to baseline levels throughout the observation period (Fig. 3).

3.6. Effect of minocycline on development of nerve injury-induced allodynia and hyperalgesia in rats

In the present series of experiments, the baseline paw withdrawal response in each test obtained on day 0 for each rat was relatively

Table 1
Effect of minocycline on exudate volume, total and differential cell counts, and TNF-α level in peritoneal exudate of acetic acid or zymosan-injected mice

Treatment	Increase in exudate volume (ml)	Total leukocytes (10^6/cavity)	PMNs (10^6/cavity)	Mononuclear cells (10^6/cavity)	TNF-α (pg/cavity)
Saline	0.00±0.00	4.21±0.61	3.38±0.41	0.51±0.08	32.33±4.56
Saline+zymosan	0.73±0.08[a]	19.65±1.68[a]	15.56±1.66[a]	2.89±0.57[a]	371.26±26.16[a]
MIN 30+zymosan	0.69±0.07	18.88±1.91	14.61±1.31	3.06±0.46	347.71±22.37
MIN 100+zymosan	0.76±0.09	19.01±2.33	16.90±0.99	2.94±0.69	366.16±39.79
V+acetic acid	0.91±0.07[a]	23.67±3.01[a]	17.66±1.12[a]	4.65±1.03[a]	298.75±28.76[a]
MIN 30+acetic acid	0.88±0.08	25.39±2.49	16.93±1.41	4.13±0.79	281.36±31.19
MIN 100+acetic acid	0.86±0.07	22.76±1.96	16.19±1.49	4.39±0.86	302.46±23.11

Minocycline (MIN; 30 or 100 mg/kg, i.p.) was administered 30 min before acetic acid (1% v/v; 0.1 ml/10 g mouse) or zymosan (2 mg/ml; 1 mg/cavity) injection. Peritoneal exudate retrieved 30 min after acetic acid or zymosan injection. The results are reported as mean±SEM for 6–8 mice/group. PMNs: polymorphonuclear cells; TNF-α: tumor necrosis factor-alpha; V: vehicle.
[a] $P<0.05$ as compared to saline-treated group.

stable and showed no significant variation. Before the chronic constriction injury, application of an innocuous cold stimulus (acetone drop) to the left or right hind paw evoked no flexor response. The mean paw withdrawal threshold to pressure was 173.66±11.33 g and 179.37±13.42 g, respectively, on day 0 before performing surgery. Following surgery, the animals 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 paw withdrawal responses to cold and mechanical stimulation in sham-operated rats remained unchanged from baseline values throughout the entire observation period. The ipsilateral paw withdrawal responses of all the vehicle-treated nerve injured rats were significantly different from that of sham-operated rats on day 4 onwards and reached steady state between days 7 and 28 after surgery indicating the development of allodynia (Fig. 4A) and hyperalgesia (Fig. 4B) in a time-dependent manner.

The development of allodynia and hyperalgesia in the ipsilateral hind paw were significantly attenuated by chronic administration of minocycline (10 or 30 mg/kg, i.p., 2 h before and once daily for 7 days post-nerve injury) in the CCI rats as compared to vehicle-treated CCI rats (Fig. 4A and B). In addition, following the termination of treatment, the ipsilateral paw withdrawal responses to cold and mechanical stimulation were significantly different from that of vehicle-treated CCI rats. Both the doses of minocycline delayed the development of cold allodynia in CCI rats on 10, 14 and 21, but not on day 28 as compared to vehicle-treated CCI rats (Fig. 4A) where as the development of mechanical hyperalgesia was delayed till day 14 post-surgery (Fig. 4B). A single dose of minocycline (10 or 30 mg/kg, i.p.) administered on day 4 (during the development of hypersensitivity) after surgery had no effect on the ipsilateral paw withdrawal responses to cold and mechanical stimulation in CCI rats (Fig. 5A and B).

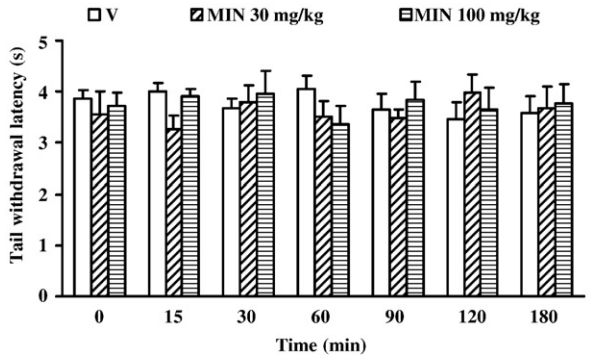

Fig. 3. Effect of minocycline treatment on tail immersion test in mice. Vehicle (V) or minocycline (MIN; 30 or 100 mg/kg, i.p.) was administered after measuring baseline responses (s) and nociceptive responses to thermal stimulation were noted in all the groups at mentioned intervals for 120 min after minocycline injection. Responses at 0 min before minocycline administration represent baseline tail withdrawal responses. Minocycline did not alter tail withdrawal responses for 2 h. Values are mean±S.E.M. (n=6–8 in each group).

3.7. Effect of minocycline on maintenance of nerve injury-induced allodynia and hyperalgesia in rats

Chronic treatment with minocycline (10 or 30 mg/kg, i.p., initiated on day 7 and continued once daily till day 28 post-nerve injury) in CCI rats did not produce any significant difference in the ipsilateral paw withdrawal responses to cold and mechanical stimulation throughout the observation period (Fig. 6A and B).

In all these experiments, systemic administration of minocycline in both the development and the maintenance paradigms had no effect

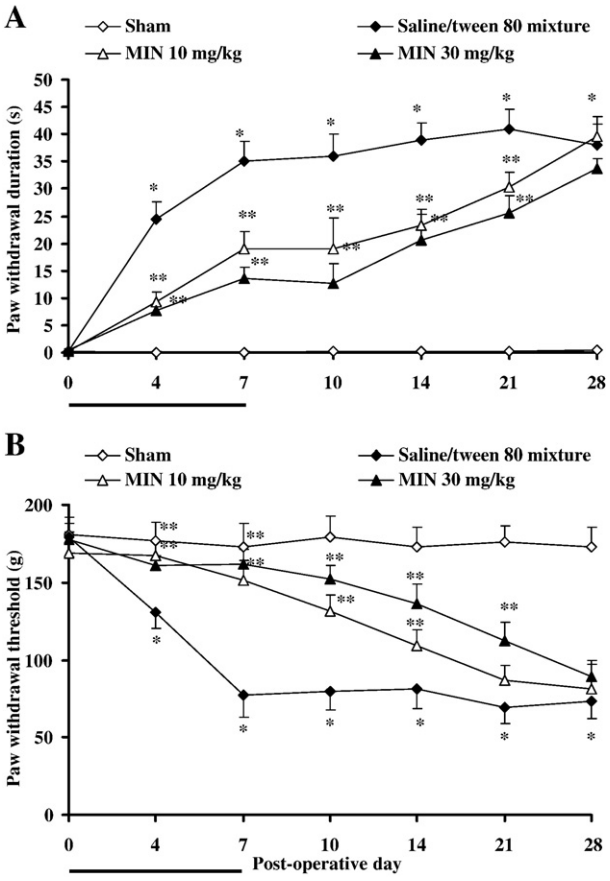

Fig. 4. Effect of minocycline pre-treatment on development of nerve injury-induced allodynia and hyperalgesia in rats. Vehicle (saline/tween 80 mixture) or minocycline (MIN; 10 or 30 mg/kg, i.p.) was initiated 2 h before surgery and continued once daily for 7 days following nerve injury. The paw withdrawal response to (A) cold (s) and (B) mechanical (g) stimulation was tested from day 0 to 28 following nerve injury. Responses on day 0 represent baseline paw withdrawal responses. The line under the X-axis indicates the duration of minocycline treatment. Values are mean±S.E.M. *$P<0.05$ vs sham-operated and **$P<0.05$ vs vehicle-treated nerve injured (CCI) animals (one-way RM-ANOVA followed by Tukey's test). (n=6–8 in each group).

on the contralateral paw withdrawal responses in these tests as compared to vehicle treatment (data not shown).

3.8. Effect of minocycline on IL-1β and TNF-α levels in the sciatic nerves of CCI rats

As the pro-inflammatory cytokines are involved in central sensitization, development and maintenance of neuropathic pain, the present work also analyzed if minocycline modulate their levels. There was marked increase in IL-1β and TNF-α level in the sciatic nerves of rats on days 14 and 28. Pre-treatment with minocycline significantly decreased, however, post-treatment although decreased the levels of the sciatic nerve IL-1β and TNF-α, but not to a significant level as compared to vehicle treatment (Table 2).

3.9. Effect of minocycline on the markers of oxidative and nitrosative stress following nerve injury

Activated microglia and pro-inflammatory cytokines following nerve injury induce generation of free radicals, which leads to oxidative and nitrosative stress, that play an important role in central sensitization, therefore, we also evaluated the effect of minocycline on the markers of oxidative and nitrosative stress. Chronic constriction injury of sciatic nerve induced a marked increase in thiobarbituric acid reactive substances and nitrite/nitrate levels, and decrease in GSH in sciatic nerves. Systemic administration of minocycline initiated before and continued during the development of neuropathic pain significantly attenuated oxidative and nitrosative stresses following nerve injury in rats. Conversely, post-treatment with minocycline, once neuropathic pain was established, had no effect on the markers of oxidative and nitrosative stress (Table 2).

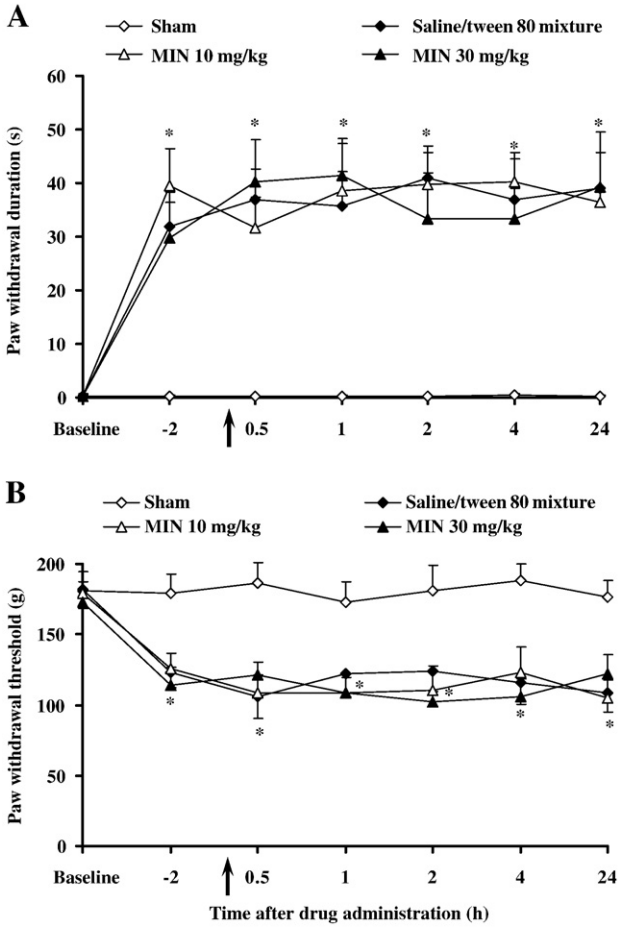

Fig. 5. Effect of single dose of minocycline on existing hypersensitivity in nerve injured rats. A single dose of minocycline (MIN; 10 or 30 mg/kg, i.p.) was administered on day 4 following nerve injury when hypersensitivity was developing and the paw withdrawal response to (A) cold (s) and (B) mechanical (g) stimulation was tested on 2 h before, from 0.5 to 4 h and 24 h after treatment. Responses on day 0 represent baseline paw withdrawal responses. The arrow under the *X*-axis indicates the time of minocycline administration. Acute treatment with minocycline had no effect on existing hypersensitivity following nerve injury. Values are mean±S.E.M. *$P<0.05$ vs sham-operated animals (one-way RM-ANOVA followed by Tukey's test). (n=6–8 in each group).

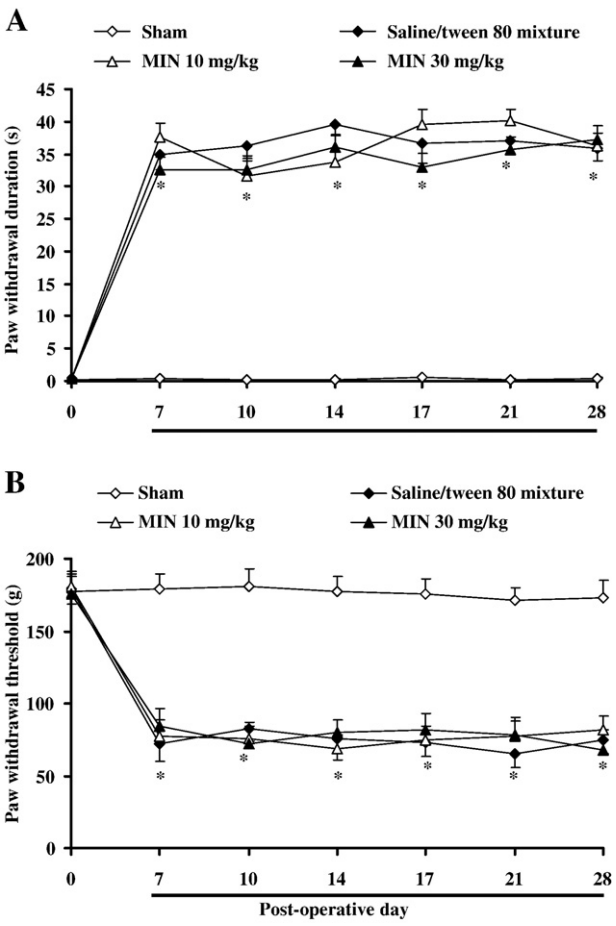

Fig. 6. Effect of minocycline post-treatment on maintenance of nerve injury-induced allodynia and hyperalgesia in rats. Vehicle (saline/tween 80 mixture) or minocycline (MIN; 10 or 30 mg/kg, i.p.) was initiated on day 7 and continued once daily for 28 days following nerve injury. The paw withdrawal response to (A) cold (s) and (B) mechanical (g) stimulation was tested from day 0 to 28 following nerve injury. Responses on day 0 represent baseline paw withdrawal responses. The line under the *X*-axis indicates the duration of minocycline treatment. Chronic treatment with minocycline did not reverse hypersensitivity once established following nerve injury. Values are mean±S.E.M. *$P<0.05$ vs sham-operated animals (one-way RM-ANOVA followed by Tukey's test). (n=6–8 in each group).

4. Discussion

In this study, we systematically investigated the effect of minocycline on peritoneal inflammation, subsequent acute pain, the tail immersion test, and neuropathic pain as well. We have observed that minocycline, which is a second-generation tetracycline, had no effect on acute peripheral inflammation and nociception, on the contrary it significantly attenuated the development of hypersensitivity following nerve injury. Further, we have also investigated the long lasting antiallodynic and antihyperalgesic effects of minocycline and its possible mechanisms involved in attenuating hypersensitivity.

Table 2
Effect of minocycline on IL-1β and TNF-α levels and the markers of oxidative and nitrosative stress in the sciatic nerve following nerve injury in rats

Treatment	Thiobarbituric acid reactive substances (μmol/g wet weight)	GSH (μg/mg wet weight)	Nitrite/nitrate (μmol/g wet weight)	IL-1β (pg/mg protein)	TNF-α (pg/mg protein)
Pre-treatment					
Sham	0.12±0.02	0.41±0.06	1.48±0.21	16.02±3.67	33.13±6.25
Vehicle	0.38±0.04[a]	0.13±0.02[a]	6.91±0.77[a]	121.33±9.56[a]	189.16±23.37[a]
MIN 10	0.21±0.02[b]	0.25±0.08[b]	4.02±0.46[b]	63.63±5.61[b]	120.28±20.15[b]
MIN 30	0.15±0.01[b]	0.36±0.07[b]	2.53±0.29[b]	47.62±6.43[b]	61.67±11.33[b]
Post-treatment					
Sham	0.13±0.02	0.37±0.07	1.8±0.26	19.96±1.93	32.91±4.23
Vehicle	0.36±0.03[a]	0.11±0.02[a]	6.62±0.69 [a]	130.13±15.33[a]	176.36±19.26[a]
MIN 10	0.34±0.05	0.13±0.03	6.76±0.93	105.36±12.19	163.02±18.15
MIN 30	0.37±0.05	0.12±0.03	6.49±0.83	96.83±14.37	151.39±12.74

These parameters were evaluated in the sciatic nerves of rats pre-treated (10 or 30 mg/kg, i.p., 2 h before and once daily for 7 days post-nerve injury) or post-treated with minocycline (MIN; 10 or 30 mg/kg, i.p., initiated on day 7 and continued once daily till day 28 post-nerve injury). The tissues were harvested on days 14 and 28 post-nerve injury in pre- and post-treated groups, respectively. The results are reported as mean±SEM for 6–8 rats/group. GSH: Reduced glutathione; IL-1β: interleukin-1beta; TNF-α: tumor necrosis factor-alpha.
[a] $P<0.05$ as compared to sham.
[b] $P<0.05$ as compared to vehicle-treated CCI (nerve injured control) group.

Either through direct release or by inducing the release of pro-inflammatory mediators such as bradykinin, histamine, TNF-α and IL-1β are involved in many aspects of inflammation, including increasing vascular permeability, cell migration, edema development, fever, and hyperalgesia (Nathan, 2002; Sherwood and Toliver-Kinsky, 2004). Resident cells such as macrophages, mast cells and lymphocytes are able to release large amounts of TNF-α and IL-1β after stimulation by acetic acid or zymosan (Ribeiro et al., 2000). Indeed, the release of TNF-α stimulates the production of IL-1β and IL-6, which in turn stimulate the production of sympathomimetic amines, prostaglandins, nitric oxide (NO), which are involved in sensitization of nociceptors (Cunha et al., 1992). In the present study, minocycline, when administered prior to nociceptive stimuli did not affect writhing behavior. Interestingly, systemic acute administration of minocycline did not reduce markers of peripheral inflammation i.e. vascular permeabililty, increase in peritoneal exudate, migration of inflammatory cells, TNF-α release, and also nociceptive responses.

In order to evaluate the effect of minocycline on acute nociception that involves spinal sensory processing, we investigated its activity in the tail immersion test. Our data show that minocycline was clearly ineffective in this test. It is unlikely that minocycline fail to reach threshold levels in spinal cord to alleviate sufficient nociceptive response in these models, because minocycline is known to readily cross the blood-brain barrier and significant neuroprotection was achieved after systemic administration (Aronson, 1980; Liu and Hong, 2003). In a recent study, systemically administered minocycline reduced carrageenan-induced paw edema and formalin-induced inflammatory pain responses (Bastos et al., 2007). Accumulating data indicate that minocycline improved percent leukocyte migration inhibition and footpad thickness which measures cell-mediated immune responses and produced a significant decrease in the paw volume in the footpad-thickness test which indicates a decrease in lymphokine production/release in chronic inflammatory pain model of rheumatoid arthritis (Reeta et al., 2002). In addition, minocycline also reduced chronic inflammatory pain in adjuvant- and collagen-induced arthritis which depends on accepted T-cell-dependent counterparts of rheumatoid arthritis. However, in the same study, it failed to reduce acute inflammation in an air-pouch system (Sewell et al., 1996). Moreover, minocycline is known to selectively inhibit microglial activation and a direct spinal application of minocycline did not change neuronal activity in naïve rats suggesting that microglia are not activated with acute stimuli (Owolabi and Saab, 2006; Tikka et al., 2001; Tikka and Koistinaho, 2001). These data along with the results of the present study suggest that minocycline modulates chronic but not acute inflammatory responses which depend on the status of peripheral inflammatory cells. Thus, one possibility is that minocycline might not be altering bradykinin and histamine release by inflammatory cells at the onset of acute inflammatory response that generally cause increased vascular permeability, maximal cellular infiltration, particularly polymorphonuclear neutrophils and macrophages. It is also plausible that minocycline had no effect on acute peripheral inflammatory and noxious stimuli within the time frame of these acute nociceptive tests.

In the present study, minocycline was administered to address its effect on the development and maintenance of hypersensitivity. The systemic chronic administration of minocycline following nerve injury significantly attenuated the hypersensitivity. It is important to note that similar, but acute, treatment with minocycline during the development of hypersensitivity, did not alter neuropathic pain. In addition, chronic administration of minocycline also failed to reverse existing hypersensitivity suggesting the differential effect of minocycline on the development and maintenance of hypersensitivity. The most striking findings of the study were those revealing that chronic administration of minocycline started prior to nerve injury delayed the development of hypersensitivity following chronic constriction injury even after termination of drug administration. Although, we did not examine microglial activation, recently, this differential pattern of glial activation has been demonstrated in various models of neuropathic pain (Nakagawa et al., 2007; Vega-Avelaira et al., 2007; Winkelstein and Deleo 2002). Further, it has been hypothesized that microglia and astrocytes have distinct role in hypersensitivity and microglial activation was observed in the induction phase with delayed activation of astrocytes (Colburn et al., 1997; Coyle, 1998; Narita et al., 2006). It has also been reported that there is a sequential activation of mitogen activated protein kinases (MAPK) in the spinal cord after nerve injury first in neurons, but only for a short period, then in microglia for many days and finally with a delay in several weeks, in astrocytes (Nakagawa et al., 2007; Zhuang et al., 2005, 2006). Interestingly, various studies reported that the phosphorylated p38MAPK in the dorsal horn was found in exclusively in microglia, but not in neurons or astrocytes (Jin et al., 2003; Tsuda et al., 2004). One possibility is that activated microglia contributes to the induction and development of exaggerated pain states and further activates various other cells, particularly astrocytes that are likely to contribute to maintaining hypersensitivity. Of particular relevance to this, it has been previously shown that inhibition of microglial activation attenuated the development, however, it had no effect on maintenance of hypersensitivity (Owolabi and Saab 2006; Raghavendra et al., 2003). Because, minocycline is without any effect on neurons and astrocytes (Tikka et al., 2001; Tikka and Koistinaho, 2001), it seems likely that the attenuation of hypersensitivity could be due to selective inhibition of microglial activation. These data along with

the present results support the proposal that activation of microglia is involved in the induction and development, but not in the maintenance of exaggerated pain.

In order to explore possible mechanisms involved in the antihyperalgesic and antiallodynic effects of minocycline in mononeuropathic rats, we quantified the level of pro-inflammatory cytokines, IL-1β and TNF-α in the sciatic nerve. Systemic chronic administration of minocycline following nerve injury but not during established hypersensitivity markedly reduced pro-inflammatory cytokines in sciatic nerve of mononeuropathic rats. Abundant evidence has shown that both astrocytes and microglial cells activation synthesize a variety of neuroexcitatory substances such as pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, and increases the expression of COX-2 and iNOS leading to the synthesis of prostaglandins and NO that potentiate pain transmission by neurons (Kreutzberg, 1996; Moalem and Tracy, 2006; Padi and Kulkarni, 2004; Padi et al., 2004b). These pro-inflammatory cytokines directly sensitize neurons and act indirectly in an autocrine or paracrine fashion to induce the synthesis and secretion of neurotransmitters that act on local neurons lead to hyperexcitable sensory states, which promote the development of hypersensitivity (DeLeo and Yezierski, 2001; Watkins et al., 2001). Therefore, the possibility that minocycline could decrease IL-1β and TNF-α release mediated by activated microglia following nerve injury can not be discarded.

Accumulating evidence indicates that oxidative and nitrosative stress is critically involved in the development and maintenance of neuropathic pain (Kim et al., 2004; Naik et al., 2006a; Park et al., 2006). Further, both invading macrophages and activated microglia have the capacity to release pro-inflammatory cytokines and free radicals. Recent studies indicated that increased mitochondrial reactive oxygen species in dorsal horn neurons also contribute to central sensitization and initial induction of allodynia which are attenuated by systemic, spinal and intracerebroventricular administration of antioxidants in neuropathic rats (Kim et al., 2004; Park et al., 2006; Twining et al., 2004). Consistent with previous studies, in the present study, minocycline attenuated oxidative stress during the development, in contrast, it had no effect on the markers of oxidative and nitrosative stress in the maintenance phase suggesting the role of activated microglia in the development of neuropathic pain. It is well documented that the neuroprotective effect of minocycline in various models of CNS injury and diseases is associated with decreasing free radical generation and subsequent oxidative and nitrosative stress (Purisai et al., 2007; Yenari et al., 2006; Zhong and Lee, 2007). Adding to this, it has also been reported that the development of neuropathic pain is dependent on the levels of endogenous antioxidant levels (Guedes et al., 2006; Naik et al., 2006b). Our results provide further evidence that delayed development of hypersensitivity following nerve injury even after termination of minocycline administration is partly a reflection of the overall anti-inflammatory effects due to decreased levels of the sciatic nerve IL-β and TNF-α and in part by modulatory role of minocycline pretreatment on oxidative stress that might be involved in attenuating and further delaying the development of hypersensitivity following nerve injury.

In conclusion, the results demonstrate that there is no effect of minocycline in acute pain due to chemical and thermal noxious stimuli. Further, the observed minocycline attenuation of development of hypersensitivity due to nerve injury is likely associated with its inhibitory effect on activated microglia, subsequent release of pro-inflammatory mediators as well as oxidative stress. We conclude that chronic treatment with minocycline started early before noxious chemical stimuli and nerve injury could prevent or at least delay the development of hypersensitivity.

Acknowledgements

This research was supported by a grant from M/S Panacea Biotec Ltd., Lalru, Punjab, India (to S.S.V. Padi). The authors are indebted to Mr. S. Kumar for his help to quantify cytokines.

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