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Minocycline attenuates the development of diabetic neuropathic pain: Possible anti-inflammatory and anti-oxidant mechanisms

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ABSTRACT

Painful neuropathy, a common complication of diabetes mellitus is characterized by allodynia and hyperalgesia. Recent studies emphasized on the role of non-neuronal cells, particularly microglia in the development of neuronal hypersensitivity. The purpose of the present study is to evaluate the effect of minocycline, a selective inhibitor of microglial activation to define the role of neuroimmune activation in experimental diabetic neuropathy. Cold allodynia and thermal and chemical hyperalgesia were assessed and the markers of inflammation and oxidative and nitrosative stress were estimated in streptozotocin-induced diabetic rats. Chronic administration of minocycline (40 and 80 mg/kg, *i.p.*) for 2 weeks started 2 weeks after diabetes induction attenuated the development of diabetic neuropathy as compared to diabetic control animals. In addition, minocyline treatment reduced the levels of interleukin-1β and tumor necrosis factor-α, lipid peroxidation, nitrite and also improved antioxidant defense in spinal cords of diabetic rats as compared to diabetic control animals. In contrast, minocycline (80 mg/kg, *per se*) had no effect on any of these behavioral and biochemical parameters assessed in age-matched control animals. The results of the present study strongly suggest that activated microglia are involved in the development of experimental diabetic neuropathy and minocycline exerted its effect probably by inhibition of neuroimmune activation of microglia. In addition, the beneficial effects of minocycline are partly mediated by its anti-inflammatory effect by reducing the levels of proinflammatory cytokines and in part by modulating oxidative and nitrosative stress in the spinal cord that might be involved in attenuating the development of behavioral hypersensitivity in diabetic rats.

1. Introduction

Diabetic neuropathy is a common complication of diabetes mellitus and characterized by spontaneous pain, hyperalgesia, allodynia, parasthesias and dysthesias. Most importantly, hyperglycemia is involved in the pathogenesis of diabetic neuropathy and other complications via complex and inter-related multiple mechanisms. Accumulating data indicate the involvement of oxidative and nitrosative stress due to generation of advanced glycation end products (Brownlee, 2005), mitochondrial dysfunction (Stevens et al., 2000), activation of nuclear factor-κB (NF-κB) (Wang et al., 2006) in the development of diabetic neuropathy (Obrosova et al., 2005). Moreover, recent studies implicate infiltration and activation of inflammatory cells (Conti et al., 2002; Yamagishi et al., 2008) as well as inflammatory cascade, particularly pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Skundric and Lisak, 2003) which through their respective receptors and signaling pathways cause neuronal hypersensitization. Current drug therapy of diabetic neuropathy is often limited and unsatisfactory due to partial effectiveness and associated side effects and further their development is hindered due to incomplete knowledge about induction and maintenance of diabetic neuropathy.

Until recently, pain has been thought to arise primarily from the dysfunction of neurons. Growing body of data implicate that the activation of non-neuronal cells (microglia and astrocytes) play an important role in central sensitization (Raghavendra et al., 2003; Hains and Waxman, 2006). Recent studies have demonstrated the activation of glial cells, particularly microglia in spinal cord (Daulhac et al., 2006; Tsuda et al., 2008), retina (Krady et al., 2005) and hypothalamus (Luo et al., 2002) in uncontrolled hyperglycemic conditions by recognizing the morphological changes in the glial cells like hypertrophy and increase in thickness and retraction of processes. Following phenotypic changes, activated microglia release variety of neuromodulators and neuroactive substances like reactive oxygen species (ROS) (Quan et al., 2007), nitric oxide (NO) and

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peroxynitrite (Li et al., 2005), prostaglandins (PGs) (Candelario-Jalil et al., 2007), and proinflammatory cytokines (IL-1β and TNF-α) (Ledeboer et al., 2005) which have been implicated directly in the induction of neuropathic pain. Glutamate has been reported to be one of the excitatory amino acid released from neurons and activate microglia (Tikka and Koistinaho, 2001). A recent study reported that the development of diabetes-induced hyperalgesia involves spinal mitogen-activated protein kinase (MAPK) activation in neurons and microglia via N-methyl-D-aspartate (NMDA) dependent mechanisms (Daulhac et al., 2006). However, the role of microglia and their pharmacological modulation is poorly understood in diabetes-induced painful neuropathy.

Minocycline, a broad spectrum, semisynthetic, and long acting tetracycline which is well absorbed and has superior tissue penetration including blood–brain-barrier (Yrjanheikki et al., 1999; Kielian et al., 2007). It is a potent inhibitor of microglial activation with no direct actions on astroglia and neurons (Ledeboer et al., 2005; Kielian et al., 2007). Further, it is well reported that anti-inflammatory action of minocycline is independent of its antibiotic effect (Kielian et al., 2007). In addition, the anti-inflammatory and neuroprotective roles of minocycline have been studied in models of peripheral and central neuropathic pain (Raghavendra et al., 2003; Hains and Waxman, 2006; Padi and Kulkarni, 2008), glutamate-induced neurotoxicity (Tikka and Koistinaho, 2001), focal and global ischemia (Yrjanheikki et al., 1999) and in many neurological diseases like Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, multiple sclerosis (Sapadin and Fleischmajer, 2006). Moreover, chemotherapeutic efficacy of minocycline treatment has also been evaluated in studies related to attenuated bone formation and collagen synthesis in experimental diabetes (Bain et al., 1997). Although, minocycline has been used as pharmacological tool to define the role of activated microglia in the induction and maintenance of peripheral neuropathy (Raghavendra et al., 2003; Ledeboer et al., 2005), no studies have examined its effect in the development of diabetic neuropathy.

Thus, the present study was designed to investigate whether minocycline decreases the development of allodynia and hyperalgesia in experimental diabetic neuropathy and whether the possible effects of minocycline are associated with decreased inflammation and oxidative stress in the spinal cord.

2. Materials and methods

2.1. Animals

Male Wistar rats (Central Animal House facility of ISF College of Pharmacy, Moga, Punjab, India) 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 food and water *ad libitum*. Animals were acclimatized to laboratory conditions before the test. All the behavioral assessments were carried between 8:00 and 16:00 h. The experimental protocols were approved by the Institutional Animal Ethics Committee and were carried out in accordance with the guidelines of the Indian National Science Academy for the use and care of experimental animals. Each animal was used for a single treatment and each group consisted of eight to ten animals. All experiments for a given treatment were performed using age-matched animals in an attempt to avoid variability between experimental groups.

2.2. Induction and assessment of diabetes in rats

Rats (weight range, 210–260 g) were intraperitoneally injected with streptozotocin (50 mg/kg) to induce type I diabetes. Age-matched control rats received, in parallel, an equal volume of citrate buffer. The induction of diabetes was confirmed by measurement of tail vein blood glucose levels using enzymatic GOD-POD (glucose oxidase peroxidase) diagnostic kit 72 h after streptozotocin injection. Only rats with blood glucose concentration in the range between 250 and 350 mg/dl 3 days after streptozotocin injection (day 1 of diabetes induction) were used for the present study. Body weight and blood glucose levels were systematically measured before and after 2 and 4 weeks of the experiment.

2.3. Chemicals and materials

Minocycline hydrochloride (Wyeth Limited, Bangalore, India), streptozotocin (Sigma-Aldrich Corporation, India) and formalin (37% formaldehyde) (SD Fine Chemicals, India) were used in this study. GOD-POD glucose estimation kit was purchased from Erba, Transasia Bio-Medicals, India. Rat IL-1β and TNF-α ELISA kits (R&D systems, MN, USA) were used to quantify cytokines. Unless stated, all other chemicals and biochemical reagents of highest analytical grade quality were used. Minocycline for *i.p.* administration was freshly prepared by solubilising in sterile normal saline. The solutions were administered 0.5 ml per 100 g rat. Formalin was diluted in sterile normal saline.

2.4. Behavioral test paradigm

2.4.1. Cold allodynia

Cold allodynia was assessed as the paw withdrawal latency (s) to thermal, non-noxious stimulus of hind paws when dipped in water bath maintained at 10 ± 0.5 °C (Padi and Kulkarni, 2004). Baseline latency of paw withdrawal to cold stimulation was established thrice, 5 min apart, and averaged. A cut-off time of 15 s was imposed to avoid potential tissue injury. A significant reduction in paw withdrawal latency (PWL) indicated allodynia.

2.4.2. Tail immersion test

Tail of the rat was immersed in warm water bath maintained at 46 ± 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. The change in the tail withdrawal latency (TWL) as compared to basal responses was calculated as a measure of hyperalgesia in all the groups and is attributed to central mechanisms (Courteix et al., 1998).

2.4.3. Thermal hyperalgesia

The development of thermal hypersensitivity associated with neuropathic pain was measured using mean PWL (s) of the rat paw when dipped in water bath maintained at 47 ± 0.5 °C. The baseline latency of paw 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 paw. The change in the PWL as compared to basal responses was calculated as a measure of hyperalgesia in all the groups (Padi et al., 2004a, 2004b).

2.4.4. Formalin-induced flinching

The rats were briefly allowed to acclimate in open Plexiglas observation chambers for 30 min before injecting nociceptive stimuli. Age-matched control and diabetic animals were gently restrained and injected with 50 µl of 2% formalin solution in normal saline subcutaneously into the plantar surface of the right hind paw with a 26-guage needle. Animals were transferred back to the chambers and nociceptive behavior was observed immediately after formalin injection. Nociceptive behavior was quantified as the number of flinches of the injected paws every 5 min, up to 60 min after injection. Formalin induced flinching behavior was biphasic. Initial acute phase (0–10 min) was followed by a relatively short quiescent period which was then pursued by a prolonged tonic response. The results were

expressed as the sum of flinching responses in phase 1 and 2 in the formalin test (Calcutt et al., 1996).

2.5. Collection of blood and tissues samples in rats

In this study, at the end of treatment schedule on day 28, blood was collected for blood glucose and the animals were euthanized by overdose of thiopental sodium (200 mg/kg, *i.p.*) immediately after behavioral assays, followed by collection of spinal cord for estimation of markers of inflammation and oxidative stress.

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. One portion of spinal cord was homogenized in homogenization buffer containing protease inhibitor. These samples were cold centrifuged and the supernatant was used for estimation of pro-inflammatory cytokines as per manufacturer's specifications. The remaining part of spinal cord was washed with sterile normal saline, weighed separately, homogenized in phosphate buffer pH 7.0 and centrifuged for 15 min at 2000 *g* to obtain the clear supernatant for the estimation of oxidative stress markers.

2.6. Pro-inflammatory cytokines

Spinal cord was isolated 2 weeks after minocycline administration (day 29) and weighed sections were homogenized in homogenization buffer. Samples were cold centrifuged and supernatant was used for estimation of IL-1β and TNF-α concentration using the quantitative sandwich enzyme immunoassay according to manufacturer's instructions (R&D systems, MN, USA). The cytokine level was determined by comparing samples to the standard curve generated from the respective kits at 450 nm and are expressed as pg per mg tissue (spinal cord).

2.7. Markers of oxidative stress

2.7.1. Lipid peroxidation

Lipid peroxidation in spinal cord was estimated colorimetrically by measuring thiobarbituric acid reactive substances by the method of Niehius and Samuelsson (1968). 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 nmole per g tissue.

2.7.2. Protein carbonylation

Protein carbonyl content in spinal cord was determined by the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dintrophenylhydrazone (Levine et al., 1990). 0.1 mL of supernatant was incubated with 0.5 ml DNPH for 60 min with subsequent precipitation of protein from the solution using 20% trichloroacetic acid. The pellet was washed after centrifugation (3400 *g*) with ethyl acetate:ethanol (1:1 vv^{-1}) mixture thrice to remove excess of DNPH. The final protein pellet was dissolved in 1.5 ml of 6 M guanidine hydrochloride and the absorbance was measured at 370 nm (UV-1700 Spectrophotometer, Shimadzu, Japan). The results were expressed as μmole per g tissue.

2.7.3. 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. 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 % activity of vehicle-treated control.

2.7.4. Nitrite

The spinal cord nitrite levels were measured by the Griess reaction (Sastry et al., 2002). 0.1 ml of supernatant was mixed with 250 μl of 1% sulfanilamide (prepared in 3 N HCl) and 250 μl of 0.1% N-naphthylenediamine 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 μmole per g tissue.

2.8. Study design

All animals were acclimatized to laboratory environment for at least 2 h before testing. Two weeks after diabetes induction, minocycline was dissolved in 0.9% saline and administered intraperitoneally (*i.p.*) at doses of 40 and 80 mg/kg and continued for further two weeks. One group of non-diabetic rats received vehicle for minocycline (age-matched control group) and another group of non-diabetic rats were administered with minocycline (*per se*; 80 mg/kg, *i.p.*). The response to behavioral nociceptive tests was assessed on days 1 (24 h after diabetes induction before starting the treatment), 14 (on the day before initiation of the treatment) followed by assessment on every third or fourth day of a week of treatment initiation (days 17, 21, 24, and 28) for the next two weeks.

2.9. Statistical analysis of data

The results are presented as mean ± S.E.M. for atleast 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. Streptozotocin injection and induction of diabetes

About 85% animals were diabetic 72 h after streptozotocin administration as indicated by blood glucose levels more than 250 mg/dl (91.53 ± 5.33 mg/dl before *vs* 306.49 ± 11.95 mg/dl after streptozotocin administration). When induced, hyperglycemia remained constant (327.13 ± 14.42 mg/dl on day 28) before behavioral assessment (Table 1).

3.2. Effect of minocycline on blood glucose levels and body weight

At the end of the experiment, diabetic rats exhibited significantly increased glucose levels compared with the control rats. Chronic administration of minocycline (40 or 80 mg/kg, *i.p.*, for two weeks) did not alter 4 week diabetic hyperglycemia as compared to that in

Table 1
Effect of minocycline (M; 40 or 80 mg/kg, *i.p.*) on body weight and blood glucose in rats.

Treatment (mg/kg)	Body weight (g)		Blood glucose (mg/dl)	
	Initial	Final	Initial	Final
V	224.17 ± 12.67	285.33 ± 10.81	94.66 ± 6.26	102.97 ± 7.15
D	235.12 ± 11.97	221.67 ± 13.39[a]	310.04 ± 12.67[a]	332.61 ± 12.65[a]
V + M 80	230.81 ± 11.72	279.17 ± 13.19	97.55 ± 5.51	103.31 ± 7.22
D + M 40	240.10 ± 12.17	225.33 ± 13.24	299.51 ± 11.04	327.03 ± 12.96
D + M 80	238.31 ± 10.30	227.51 ± 10.14	309.92 ± 12.16	321.77 ± 17.65

D: diabetic control; V: vehicle control. Values are mean ± S.E.M.
[a] $P<0.05$ *vs* V.

vehicle-treated diabetic group (Table 1). A marked decrease in the body weights of streptozotocin -treated rats was observed on day 28 when compared with non-diabetic control animals (Table 1). Minocycline (40 or 80 mg/kg, *i.p.*) treatment had no effect on these values.

3.3. Effect of minocycline on cold allodynia

At the end of week 4, diabetic animals exhibited a significant decrease in pain threshold from non-noxious stimuli compared to age-matched, vehicle-treated group (Fig. 1(A), $p<0.05$). There was no significant difference between PWLs during the entire observation period following minocycline (*per se;* 80 mg/kg, *i.p.*, for 2 weeks) administration in non-diabetic rats (Fig. 1(A)). Systemic administration of minocycline (40 or 80 mg/kg, *i.p.*, for 2 weeks) after two weeks of untreated diabetes significantly prevented the development of hypersensitivity to cold stimulus in diabetic rats as compared to vehicle-treated diabetic group (Fig. 1(A)).

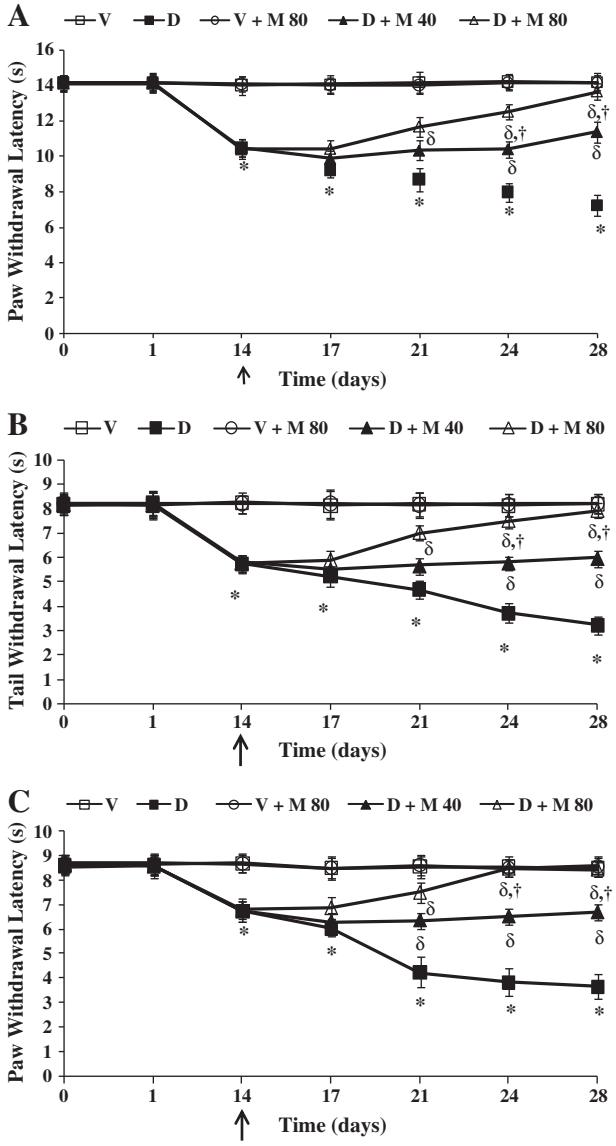

Fig. 1. Effect of minocycline (M; 40 or 80 mg/kg, i.p.) on (A) paw withdrawal latency to cold stimuli in rats indicative of allodynia; (B) tail withdrawal latency to thermal stimuli and (C) paw withdrawal latency to thermal stimuli in rats indicative of hyperalgesia in development of diabetic neuropathy. Arrow indicates responses 24 hrs before the day of treatment initiation. D: diabetic control; M: minocycline; V: vehicle control. Values are mean ± S.E.M. $^{*}P<0.05$ *vs* V; $^{\delta}P<0.05$*vs* D; $^{\dagger}P<0.05$ *vs* D + M 40.

3.4. Effect of minocycline on thermal hyperalgesia

The threshold for thermal hyperalgesia was significantly decreased at day 14 and continued to develop up to day 28 after streptozotocin injection as compared to vehicle treated age-matched non-diabetic animals. Chronic administration of minocycline (*per se;* 80 mg/kg, *i.p.*, for 2 weeks) was without any effect on TWLs (Fig. 1(B)) and PWLs (Fig. 1(C)) in non-diabetic rats whereas, similar administration of minocycline (40 or 80 mg/kg, *i.p.*, for 2 weeks) markedly prevented the development of hyperalgesia in diabetic rats as compared to vehicle-treated diabetic rats (Fig. 1(B) and (C)).

3.5. Effect of minocycline on formalin-induced flinching

All the rats subcutaneously challenged with formalin into hind paw showed characteristic biphasic response with an early phase 1 and a late phase 2 separated by a quiescent phase. No significant difference in the sum of flinches counted in phase 1 was observed between age-matched control and diabetic animals on day 28. 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 28 (Fig. 2). Minocycline (*per se;* 80 mg/kg, *i.p.*, for 2 weeks) administration did not alter nociceptive response in phase 1 and phase 2 (Fig. 2) of the formalin test in normal rats on day 28 whereas systemic administration of minocycline (80 mg/kg, *i.p.*, for 2 weeks) significantly decreased formalin-induced nociceptive behavior in phase 2 but not phase 1 as compared to vehicle treatment in diabetic rats.

3.6. Effect of minocycline on IL-1β and TNF-α levels in the spinal cord of diabetic rats

The concentration of spinal cord IL-1β and TNF-α (Fig. 3) was significantly elevated in four week diabetic rats when compared to that of vehicle-treated age-matched control rats. Chronic administration of minocycline (*per se*; 80 mg/kg, *i.p.*, for 2 weeks) had no effect on spinal cord IL-1β and TNF-α levels in non-diabetic animals. However, the concentration of these cytokines was significantly lower in diabetic rats that had been treated with minocycline (40 or 80 mg/kg, *i.p.*, for 2 weeks), (Fig. 3).

3.7. Effect of minocycline on the markers of oxidative and nitrosative stress

Free radicals generated by activated microglia and pro-inflammatory cytokines in response to hyperglycemia lead to oxidative and nitrosative stress, that play an important role in central sensitization. Thus we also evaluated the effect of minocycline on the markers of oxidative and nitrosative stress. Chronic hyperglycemia induced a marked increase in

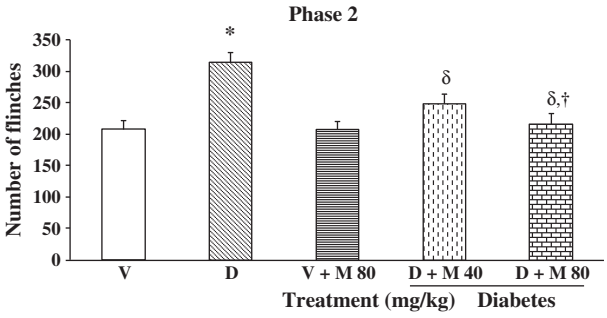

Fig. 2. Effect of minocycline (M; 40 or 80 mg/kg, *i.p.*) on Phase 2 nociceptive responses in the formalin test indicative of hyperalgesia in development of diabetic neuropathy. D: diabetic control; M: minocycline; V: vehicle control. Values are mean ± S.E.M. $^{*}P<0.05$ *vs* V; $^{\delta}P<0.05$*vs* D; $^{\dagger}P<0.05$ *vs* D + M 40.

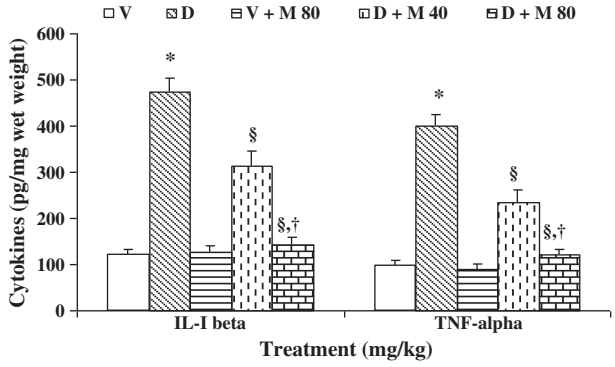

Fig. 3. Effect of minocycline (M; 40 or 80 mg/kg, *i.p.*) on spinal cord interleukin (IL)-1β and tumor necrosis factor (TNF)-α in rats. D: diabetic control; M: minocycline; V: vehicle control. Values are mean ± S.E.M. $^{*}P<0.05$ *vs* V; $^{§}P<0.05$*vs* D; $^{†}P<0.05$ *vs* D + M 40.

thiobarbituric acid reactive substances, protein carbonylation and nitrite levels, and decrease in the activity of superoxide dismutase in spinal cord. Systemic administration of minocycline during the development of diabetic neuropathic pain significantly attenuated oxidative and nitrosative stresses. On the contrary, treatment with minocycline (*per se;* 80 mg/kg, *i.p.*, for 2 weeks) in non-diabetic animals had no effects on the markers of oxidative and nitrosative stress as compared to vehicle-treated age-matched control group (Table 2).

4. Discussion

In the present study, we investigated the effect of minocycline, an inhibitor of microglial activation on the development of diabetic neuropathy. The assessment of hypersensitivity was done using behavioral assays of neuropathic pain that include tail and paw withdrawal latencies to noxious heat stimuli (thermal hyperalgesia), formalin-induced exaggerated flinching responses (chemical hyperalgesia), and PWLs to non-noxious cold stimuli (cold allodynia). The results of the present study demonstrate that (1) pre-emptive administration of minocycline attenuated the development of painful diabetic neuropathy (2) that is associated with decreased levels of proinflammatory cytokines and oxidative stress in the spinal cord of diabetic animals, and supports that (3) spinal microglia become activated in hyperglycemic condition leading to elevation of proinflammatory cytokines and oxidative stress.

Altered perception threshold with increased sensitivity to noxious and non-noxious stimuli are observed in large proportions of diabetic patients. In the tail-flick test, the delay in removing the tail from noxious stimuli is evaluated reflecting the activity of simple spinal reflex arc (Calcutt, 2001; Ilnytska et al., 2006). Moreover, the paw withdrawal responses to noxious thermal stimuli (hyperalgesia) reveal supraspinal sensory processing (Calcutt, 2001; Obrosova et al., 2005). In the present study, a marked thermal hyperalgesia in both the tests was observed in diabetic animals which were corrected by minocycline. In line with previous studies, in the present study also, diabetic animals developed cold allodynia which was corrected by treatment with minocycline.

In the formalin test, phase 1 responses reflect acute nociceptive pain similar to the thermal nociceptive tests whereas phase 2 responses are attributed to the combination of on-going inflammatory-related afferent input from peripheral tissue and functional changes in the dorsal horn of the spinal cord. In the present study, diabetic animals exhibited exaggerated flinching responses in phase 2, but not in phase 1, of the formalin test, which were blunted by administration of minocycline. Thus, it is clear from the behavioral studies that minocycline attenuated the development of diabetic neuropathy and suggest the involvement of activated microglia in nociceptor hypersensitization in diabetes.

It is well known that minocycline, a second generation tetracycline, inhibits the activation and proliferation of microglia in the central nervous system. Further, it crosses blood–brain-barrier readily and provides significant neuroprotection and antinociceptive effects after systemic administration (Yrjanheikki et al., 1999; Raghavendra et al., 2003; Kielian et al., 2007; Padi and Kulkarni, 2008). However, minocycline had no effect on acute nociceptive tests such as tail flick and hot plate which further supports that ramified microglia are not activated within the time frame of these assays in normal animals (Padi and Kulkarni, 2008). Moreover, direct spinal application of minocycline did not alter neuronal activity in naive rats suggesting that microglia are not activated in normal physiological state and on application of acute nociceptive stimuli (Owolabi and Saab, 2006). Consistent with previous reports, in the present study minocycline had not affected basal responses in non-diabetic animals indicating that the effects of minocycline in diabetic animals are not due to analgesic or hypoalgesic effects. It has been reported that spinal administration of activated microglia produced hypersensitivity, but not quiescent microglia and activated astroglia which indicated that activated microglia are involved in spinal sensitization (Narita et al., 2006). Because, minocycline is without any effect on neurons and astrocytes (Tikka and Koistinaho, 2001; Ledeboer et al., 2005; Kielian et al., 2007), it seems likely that the antihyperalgesic and antiallodynic effects of minocycline in attenuating behavioral hypersensitivity could be attributed to its ability to suppress the activation of microglia during the course of disease state, a property which is distinguished from its antibiotic property (Kielian et al., 2007).

It is well known that diabetic neuropathic pain is dependent on the levels of pro-inflammatory cytokines and the state of oxidative stress. Therefore, in the present study we have estimated the markers of inflammation and oxidative stress in order to gain an insight into antihyperalgesic and antiallodynic effects of minocycline. It is well known that hyperglycemia in diabetes is associated with cytokine release, which promotes the splitting of myelin and assisting the demyelinating process (Conti et al., 2002). Various preclinical and clinical studies have reported that diabetic neuropathy is associated with significant increase in proinflammatory cytokines (Skundric and Lisak, 2003). Most importantly, the source for these cytokines in the spinal cord is activated glial cells or alternatively, that activated microglia release substance(s) that, in turn causes the release of these cytokines from other cell type(s). Accumulating data indicate that TNF-α and IL-1β (Tangpong et al., 2008), ROS (Min et al., 2003), peroxynitrite (Ndengele et al., 2008) directly activate microglia.

Moreover, cytokine release is also responsible for causing neuropathic pain (Watkins and Maier, 2003; Wang et al., 2006). Consistent with previous reports, we also observed significant increase in spinal IL-1β and TNF-α levels in the development of diabetic neuropathy which were markedly attenuated by minocycline treatment (Raghavendra et al., 2003; Ledeboer et al., 2005). It has been

Table 2
Effect of minocycline (M; 40 or 80 mg/kg, i.p.) on TBARS, protein carbonylation, SOD activity and nitrite levels in spinal cord of rats.

Treatment (mg/kg)	TBARS (nmole/g tissue)	Protein carbonyl (μmole/g tissue)	% SOD activity	Nitrite (nmole/g tissue)
V	54.43 ± 3.70	1.86 ± 0.06	99.94 ± 2.31	119.84 ± 12.19
D	152.98 ± 5.36[a]	4.52 ± 0.16[a]	49.53 ± 2.18[a]	411.64 ± 28.31[a]
V + M 80	58.32 ± 4.61	1.81 ± 0.05	101.01 ± 2.26	121.33 ± 10.07
D + M 40	124.07 ± 7.93[b]	3.72 ± 0.17[b]	66.45 ± 3.60[b]	258.45 ± 34.16[b]
D + M 80	86.92 ± 4.71[b,c]	2.25 ± 0.17[b,c]	88.41 ± 2.29[b,c]	142.85 ± 20.31[b,c]

D: diabetic control; M: minocycline; SOD: Superoxide dismutase; TBARS: thiobarbituric acid reacting substances; V: vehicle control. Values are mean ± S.E.M.
[a] $P<0.05$ *vs* V.
[b] $P<0.05$ *vs* D.
[c] $P<0.05$ *vs* D + M 40.

reported that minocycline inhibited microglial activation without affecting astroglia and neurons and reduced expression and release of pro-inflammatory cytokines in spinal cord of neuropathic rats (Raghavendra et al., 2003; Ledeboer et al., 2005). In addition, proinflammatory cytokines release lead to accumulation of free radicals (Leite et al., 2007) and activate enzymes like COX-2 and iNOS, further releasing PGs and NO, well known mediators involved in spinal hypersensitization (Thacker et al., 2007). Although NF-κB was not been estimated in the present study, however, evidence exist that minocycline prevents the degradation of inhibitory subunit of IκB, thereby reduces NF-κB translocation to nucleus and its activation, which regulates transcription of proinflammatory cytokines, COX-2 and iNOS, and that is responsible for anti-inflammatory and neuroprotective properties (Yrjanheikki et al., 1999; Tikka and Koistinaho, 2001; Nikodemova et al., 2006). These data along with the results of the present study suggest that minocycline decreases proinflammatory cytokine levels that are involved in attenuating diabetic neuropathy.

Biochemical changes occurring during diabetes disrupt the functions of mitochondria leading to the increased generation of ROS and decreased antioxidant defenses in the tissues of diabetic animals (Stevens et al., 2000; Ho et al., 2006). Indeed, activated microglial cells in the CNS are an important source of free radicals (Candelario-Jalil et al., 2007), express iNOS and produce high levels of NO (Li et al., 2005) in response to a wide variety of proinflammatory and degenerative stimuli. Nitrosative and oxidative stress have been implicated in the development and maintenance of diabetic neuropathy (Stevens et al., 2000; Obrosova et al., 2005; Drel et al., 2007). Recent studies indicated that increased mitochondrial ROS in dorsal horn neurons and spinal cord also contribute to central sensitization and development of diabetic neuropathy which are attenuated by administration of antioxidants glutathione, α-lipoic acid, taurine (Stevens et al., 2000; Ho et al., 2006).

In the present study, diabetic rats had shown increased nitrite and decreased activity of SOD in the spinal cord which were improved by minocycline. In addition, minocycline reduced oxidation of lipids and proteins and improved antioxidant defenses in the spinal cord of diabetic neuropathic rats. Previous studies reported that minocycline attenuates the expressions of iNOS and subsequent NO production (Yrjanheikki et al., 1999; Tikka and Koistinaho, 2001). Abundant evidence has shown that 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 (Kraus et al., 2005). Moreover, minocycline has been revealed to scavenge superoxide (Yenari et al., 2006) and peroxynitrite (Whiteman and Halliwell, 1997) in glial cells due to its direct interaction with free radicals and hence supports its antioxidant action. Intriguingly, minocycline had no effect on the markers of oxidative stress in non-diabetic animals. Thus, based on the results, the present study provides evidence that attenuation of hypersensitivity in diabetes by minocycline is accompanied by reduced oxidative stress in the spinal cord and further suggesting the role of ROS and reactive nitrogen species in the activation of microglia and vice versa in the development of diabetic neuropathy.

In addition to direct actions on microglia, it is plausible that pleiotropic effects of minocycline might also be involved in reducing the development of neuropathic pain. It has been shown that minocycline exert anti-apoptotic effects by inhibiting the cytochrome c release from mitochondria (Teng et al., 2004) and inhibiting caspase-dependent (caspase-1 and 3) and -independent pathways of neuronal death (Wang et al., 2003). Moreover, minocycline has been reported to inhibit the activation of PARP-1 directly in the neurons (Alano et al., 2006). Apart from this, it also interfere with the activation of protein kinase C which is involved in activation of MAPK (Shigemoto-Mogami et al., 2001). Adding to this, a part of neuroprotective activity of minocycline is also associated with inhibition of p38 MAPK activation (Tikka and Koistinaho, 2001) and inhibition of IL-1β converting enzyme (ICE) (Yrjanheikki et al., 1999; Tikka and Koistinaho, 2001) that contributes to inhibition microglial activation.

In conclusion, the results demonstrate that minocycline attenuates the development of hyperalgesia and allodynia in streptozotocin-model of diabetic neuropathic pain probably by inhibiting microglial activation and partly by decreasing the inflammation and oxidative stress in the spinal cord. Additionally, provided its ease to cross blood–brain-barrier, good oral bioavailability, low incidence of adverse events in clinical studies and proven effectiveness in animal models of acute and chronic pain, minocycline could be evaluated for its effectiveness in diabetes-induced neuropathic pain. With growing interests over its possible usefulness in neuroprotection, our data suggests that minocycline worth additional studies as a possible therapeutic agent for treatment of diabetic neuropathy.

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