Minocycline through attenuation of oxidative stress and inflammatory response reduces the neuropathic pain in a rat model of chronic constriction injury

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ARTICLE INFO	ABSTRACT
	Objective(s): Several lines of evidence showed that minocycline possesses antioxidant and anti-inflammatory properties. This study aimed to demonstrate the effects of minocycline in rats subjected to chronic constriction injury (CCI). ***Materials and Methods:*** In this study four groups (n = 6–8) of rats were used as follows: Sham, CCI, CCI + minocycline (MIN) 10 mg/Kg (IP) and CCI + MIN 30 mg/Kg (IP). On days 3, 7, 14, and 21 post-surgery hot-plate, acetone, and von Frey tests were carried out. Finally, Motor Nerve Conduction Velocity Evaluation (MNCV) assessment was performed and spinal cords were harvested in order to measure tissue concentrations of TNF_α, IL-1β, Glutathione peroxidase (GPx), Superoxide dismutase (SOD) and Malondialdehyde (MDA). Extent of perineural inflammation and damage around the sciatic nerve was histopathologically evaluated. ***Results:*** Our results demonstrated that CCI significantly caused hyperalgesia and allodynia twenty-one days after CCI. MIN attenuated heat hyperalgesia, cold and mechanical allodynia and MNCV in animals. MIN also decreased the levels of TNF_α and IL-1β. Antioxidative enzymes (SOD, MDA, and GPx) were restored following MIN treatment. Our findings showed that MIN decreased perineural inflammation around the sciatic nerve. According to the results, the neuropathic pain reduced in the CCI hyperalgesia model using 30 mg/kg of minocycline. ***Conclusion:*** It is suggested that antinociceptive effects of minocycline might be mediated through the inhibition of inflammatory response and attenuation of oxidative stress.

Introduction

Neuropathic pain (NP) is caused by several factors with impairment in nerve function. The pathophysiology of pain is relatively complex and involves the central and peripheral mechanisms. Alteration in the ion channel expression, neurotransmitter release, and pain pathways are involved in the pathophysiology of pain (1). NP consists of unpleasant sensations of burning, tingling, and increased sensitivity towards the hyperalgesia and allodynia (2). When the tissue is destroyed, an inflammatory reaction leads to the activation of the pain pathway (3).

It is known that both hyperalgesia and allodynia coexist in both, the inflammatory and neuropathic pain (4).

It has been well-documented that oxidative stress contributes to the pathophysiology of NP (5). In this regard, it is demonstrated that reduction in antioxidant protection, increase in oxidant generation, and loss of the capacity of repair from oxidative damage are involved in the NP consequence of oxidative stress (6).

Minocycline is a broad-spectrum and long-acting antibiotic and also possesses anti-inflammatory and antioxidant effects (7, 8). It has been shown that minocycline acts as an inhibitor of microglial activation

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(9) and also exerts neuroprotective effects in the experimental model of brain ischemia (10, 11). Previous studies have demonstrated that systemic injection of minocycline reduces the microglia activation and consequently diminishes the production of the inflammatory cytokines in the CNS (12, 13). Ample evidence showed that minocycline protects neurons against impairment due to stroke and traumatic injury (14, 15). It is well-accepted that minocycline possesses antioxidant property (16, 17). Some researchers suggest that the neuroprotective effects of minocycline are via mitochondrial action. In this regard, minocycline executes its neuroprotective effect via inhibition (direct) of MPT (mitochondrial permeability transition) pores cytochrome *c* release (18, 19).

The aim of this study is to investigate the protective effect of different doses of minocycline on NP considering behavioral tests and antioxidant and anti-inflammatory properties in a rat model of chronic constriction injury.

Materials and Methods

Animals

Thirty-two rats (male adult Sprague–Dawley) weighing 200–250 g were purchased and housed in standard conditions (4 rats per cage) i.e. temperature 23±2 °C, humidity of approximately 50%, and 12-hr light/dark cycle (9 a.m. - 9 p.m. lights on) (20). The animals had free availability of water and food.

Experimental design

Minocycline (Sigma, St. Louis, MO, USA) was suspended in distilled water. Pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA) was used for anesthesia. Animals were divided randomly into four experimental groups (n= 8 rats per group): 1: Sham-operated (Sh), 2: CCI saline-treated (CCI), 3: CCI + Minocycline (MIN) 10 mg/kg intraperitoneal (IP), 4: CCI + MIN 30 mg/kg (IP). Minocycline was injected one day before surgery and continued daily for 21 days post-ligation. Doses of minocycline were chosen based on previously published study (21-23). Regarding these studies, we performed a pilot study and selected two doses of 10 and 30 mg/kg of minocycline.

Surgery

To induce sciatic nerve injury the CCI model of NP was performed (24). The surgical procedure was performed under pentobarbital Na (60 mg/kg) anesthesia. The left sciatic nerve was exposed and three ligations were put around the nerve. The space between two adjacent ligatures was 1 mm. In sham rats, the same surgical procedure was done, but not ligated.

Thermal stimulation tests

Behavioral tests were recorded on days 3, 7, 14, and 21 after ligation. The experiments were started with the heat hyperalgesia stimulation test and terminated with mechanical allodynia. 120 min was considered as the interval time between two tests.

Heat hyperalgesia stimulation (hot-plate test):

Eddy's hot-plate, as an index of thermal hyperalgesia, was used to study the thermal nociceptive threshold by keeping the temperature at 52.5 ± 3.0 °C. Animals were placed on the hot-plate and withdrawal latency, with respect to the licking of the hind paw, was recorded in seconds. The cut-off time of 15 sec was maintained (25).

Cold allodynia (acetone test):

In cold allodynia test, the rat's hind paw was placed over a wire mesh and 100 µl of acetone was sprayed onto its surface without touching the skin. The response to acetone was noted in 20 sec and was scored according to the criteria described by Kukkar and Singh as follows: 0: no reflex; 1: quick stamp, flick or withdrawal of the paw; 2: repeated flicking or prolonged withdrawal; and 3: repeated flicking with licking of the paw. In this test, acetone was used three times, every 5 min. The score range was defined from zero to nine (26).

Mechanical Allodynia (von Frey Test)

Briefly, the rats were placed in a cage (20 * 20 * 10 cm) whose floor was made of meshed metal wire. A chain of von Frey filament stimuli was delivered in an arising order of forces to the central section of the plantar hind paw skin (three times repeatedly). Stimulation of the plantar hind paw due to common locomotors behavior was ignored. The sequence of stimuli was stopped when the rat responded with immediate flinching or licking of the hind paw skin (27).

Motor Nerve Conduction Velocity Evaluation (MNCV)

MNCV recordings were carried out 3 weeks after CCI. On day 21, animals were anesthetized by intraperitoneal injection of pentobarbital Na (60 mg/kg, Sigma-Aldrich). To expose the sciatic nerve, the skin and muscles were disjointed layer by layer. MNCV was assessed by inducing a compound action potential from the stimulating electrode at the proximal end and recording from the recording electrode at the distal end of the injury site (28)

Enzyme-linked immunosorbent assays (ELISA)

TNF_α, IL-1β, GPx, SOD, and MDA levels were assessed in the spinal cord samples. For this purpose, the spinal cord between the L4-L5 segments was isolated immediately after behavioral measurements. Homogenate tissues were prepared with 0.9% saline using glass homogenate and then centrifuged at 2500 r/min for 10 min. Supernatant (10%,w/v) was used for ELISA assay (29, 30).
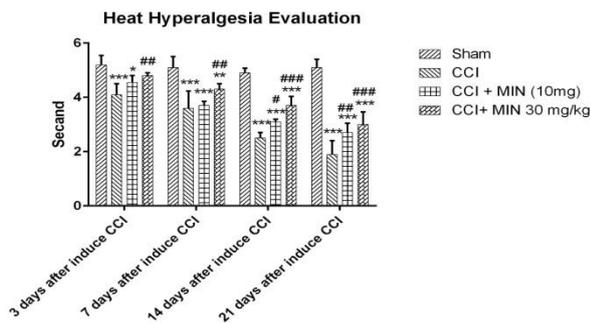
Figure 1. The effect of minocycline administration on CCI-induced heat hyperalgesia, assessed using the hot-plate test. Rats were treated with vehicle or minocycline at doses of 10 and 30 mg/kg once daily for a period of 21 days. The data were shown as mean ± SEM. ** *P< 0.01* vs Sham, ****P< 0.001* vs Sham, *#P< 0.05* vs CCI, ## *P< 0.01* vs CCI, and ### *P< 0.001* vs CCI

Figure 2. The effects of minocycline administration on CCI- induced cold allodynia assessed using the acetone test. Rats were treated with vehicle or minocycline at doses of 10 and 30 mg/kg once daily for a period of 21 days. The data were shown as mean ± SEM. *** *P< 0.001* vs Sham, # *P< 0.05* vs CCI, and ### *P< 0.001* vs CCI

Histologic evaluation

On day 21, sciatic nerves were dissected. Histological studies were performed according to the described protocol. For histological examination, samples of sciatic nerve were cut into segments of 5-micrometer sections and stained with hematoxylin and eosin. All samples were analyzed for perineural inflammation and according to protocol-defined guidelines (31).

Statistical analysis

Data were expressed as mean ± SEM. Two-way analysis of variance (ANOVA) followed by Tukey's post hoc test using the Graph-pad prism software (ver. 6) was done for data analysis. A P-value<0.05 was considered statistically significant.

Results

Minocycline decreased heat hyperalgesia

Results obtained from the hot-plate test (Figure 1) showed that CCI significantly decreased the pain threshold in days 3, 7, 14, and 21 (*P*<0.001, for all days) after the operation. Following treatment with minocycline 10 mg/kg pain threshold significantly increased when compared with the CCI group in days 14 (*P*<0.05) and 21 (*P*<0.01) post-surgery. Our findings from administration of minocycline 30 mg/kg showed that pain sensitivity decreased in days 3 (*P*<0.05), 7 (*P*<0.01), 14 (*P*<0.001), and 21 (*P*<0.001) post operation in comparison with CCI counterparts. Furthermore, minocycline at dose 30 mg/kg in days 7 and 14 post induction of NP decreased sensitivity in comparison with minocycline 10 mg/kg (P<0.05).

Minocycline decreased cold allodynia scores

Results obtained from acetone test (Figure 2) revealed that induction of CCI significantly increased allodynia scores in days 3 (*P*<0.05), 7, 14, and 21 after surgery in comparison with sham groups (*P*<0.001). *Post hoc* analysis showed that administration of

minocycline (10 and 30 mg/kg) significantly reduced allodynia scores when compared with CCI counterparts in days 7 (*P*<0.05), 14 and 21 post operation (*P*<0.001).

Minocycline increased latency to mechanical allodynia

Our results from von ferry test (Figure 3) demonstrated that CCI operation significantly increased sensitivity to mechanical allodynia in days 7 (*P*>0.05), 14 (*P*>0.001), and 21 (*P*>0.001) post induction when compared with related sham counterparts. Administration of minocycline at a dose of 30 mg/kg increased latency to mechanical stimulation at day 21 post-CCI operation in comparison with CCI groups (*P*<0.05).

Effect of minocycline on MNCV

Neural conduction was reduced in the CCI group relative to the sham group at the 21st day after injury (*P*<0.01), indicating that neural conduction was diminished (Figure 4). MNCV values began to recover by 3 weeks in 10 mg/kg and 30 mg/kg of minocycline treatment groups (*P*< 0.05, *P*< 0.1, respectively).
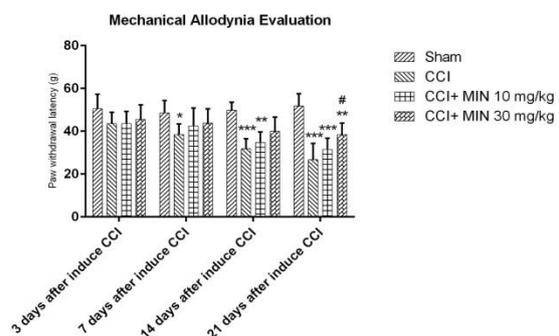
Figure 3. The effects of minocycline administration on CCI-induced mechanical allodynia assessed using the von Frey test. Rats were treated with vehicle or minocycline at doses of 10 and 30 mg/kg once daily for a period of 21 days. The data were shown as mean ± SEM. ** *P< 0.01* vs Sham, *** *P< 0.001* vs Sham, and ### *P< 0.001* vs CCI
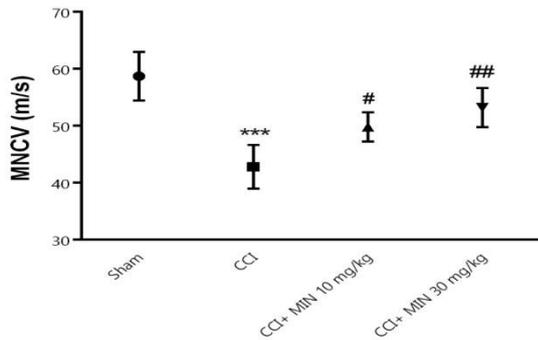
Figure 4. The effects of minocycline administration on MNCV in the sciatic nerve. Rats were treated with vehicle or minocycline at doses of 10 and 30 mg/kg once daily for a period of 21 days. The data were shown as mean ± SEM; *** $P< 0.001$ vs Sham, # $P< 0.05$ vs CCI, and ### $P< 0.001$ vs CCI

Minocycline decreased levels of inflammatory cytokines

Our findings revealed that levels of TNF-α and IL-1β significantly increased in the 21st day post-surgery in comparison with their sham counterparts ($P<0.001$ for both). Moreover, treatment with dose 30 mg/kg of minocycline decreased the concentration of IL-1β when compared with the CCI group ($P<0.05$). Furthermore, minocycline at doses 10 and 30 mg/kg significantly decreased the level of TNF-α in comparison with the CCI group ($P<0.001$ for both) (Figure 5).
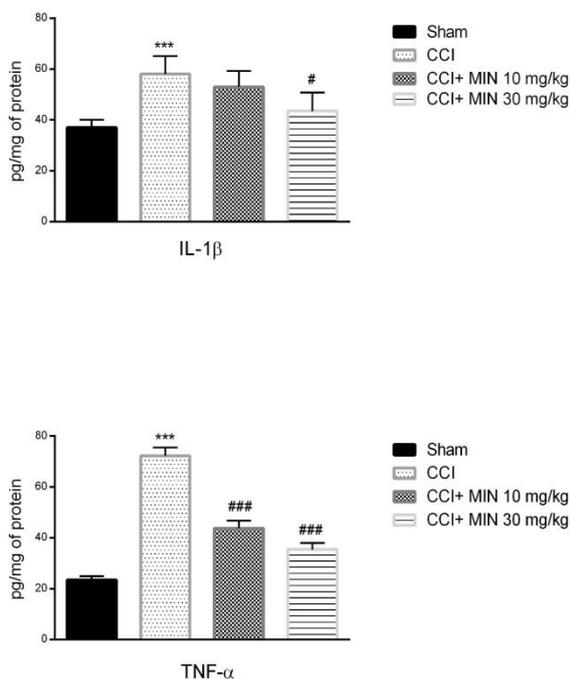
Figure 5. The effects of minocycline administration on IL-1β and TNF-α in the spinal nerve. Rats were treated with vehicle or minocycline at doses of 10 and 30 mg/kg once daily for a period of 21 days. The data were shown as mean ± SEM; *** $P< 0.001$ vs Sham, # $P< 0.05$ vs CCI, and ### $P< 0.001$ vs CCI
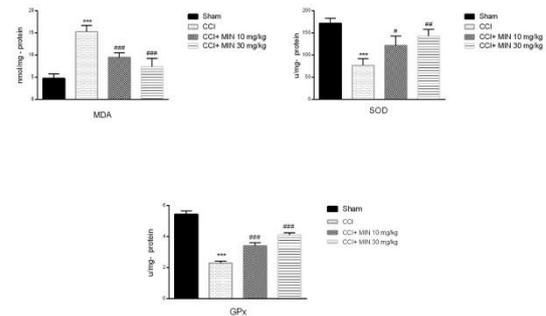
Figure 6. The effects of minocycline administration on MDA, SOD, and GPx levels in the spinal nerve. Rats were treated with vehicle or minocycline at doses of 10 and 30 mg/kg once daily for a period of 21 days. The data were shown as mean ± SEM, *** $P< 0.001$ vs Sham, # $P< 0.05$ vs CCI, ## $P< 0.01$ vs CCI, and ### $P< 0.001$ vs CCI

Effect of minocycline on oxidative markers

Measurement of concentration of markers relevant to the oxidative/anti-oxidative state using ELISA (Figure 6) showed that CCI led to increase in the level of MDA and decrease in the levels of SOD as well as GPx ($P<0.001$ for both). Minocycline (10 and 30 mg/kg) significantly decreased the levels of MDA ($P<0.001$ for both), increased the levels of SOD ($P<0.05$ and $P<0.01$, respectively), and glutamine peroxidase ($P<0.001$ for both) compared to the CCI group.

Minocycline decreased perineural inflammation around the sciatic nerve

Figure 7 shows that there are normal structural in the sciatic nerve of sham animals. Moreover, histological results from CCI groups showed that there was extensive perineural inflammation around the sciatic nerve (score=3). However, administration of minocycline (30 mg/kg) attenuated CCI induced inflammation ratio around the sciatic nerve (score = 1). Furthermore, nerve injury was not detected in the sciatic nerve of any group (Figure 7).
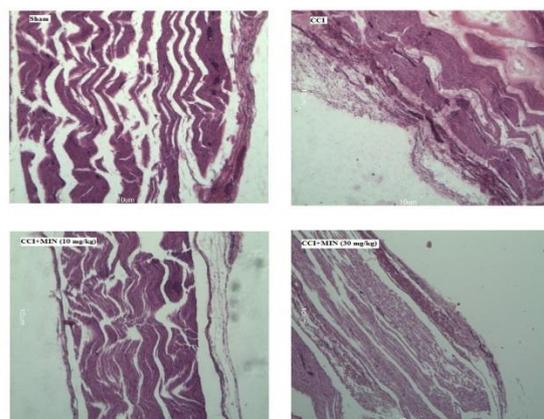
Figure 7. Histological and morphological studies in the sciatic nerve at 21 days after CCI induction. In untreated CCI rats, the sciatic nerve has diffuse areas of moderate to marked edema/cellular infiltrate. In the MIN-treated (30 mg/kg) CCI rats, the finding is small focal areas of mild edema and/or cellular infiltrate

Discussion

CCI model is a valid model used to induce NP (32). In this study, we demonstrated that chronic constriction injury induced NP within twenty-one days after the operation. Our results showed that treatment with minocycline (10 and 30 mg/kg) for a 21 day period significantly improved the behavioral changes induced by CCI, including heat hyperalgesia, cold and mechanical allodynia, and MNCV. Furthermore, minocycline attenuated inflammatory cytokines as well as oxidative stress markers. Also, we observed that administration of minocycline significantly decreased perineural inflammation around the sciatic nerve. Our findings demonstrated that optimum result were obtained from 30 mg/kg of minocycline.

It has been determined that minocycline exerted antioxidant effects and mitigated the glial cells damage due to oxidative/nitrosative stress (33, 34). Conversely, in another study, it was found that minocycline possessed some beneficial effects on the oxidative stress in diabetic animals (35). The anti-inflammatory and neuroprotective properties of minocycline have been studied. In this regards, it is determined that minocycline is effective in Parkinson's disease (36), Alzheimer's disease (37), and also in a genetic mouse model of retinitis pigmentosa (38). El-Shimy *et al.* showed that minocycline reduced the inflammatory response and exerted protective effects in nerve cells against nerve toxins (39).

Minocycline reduced the activity of the MAPK pathway in microglia (40). It is found that minocycline acted as an anti-apoptotic agent (41) and protected neurons from caspase activation through decreased Ca+ absorption and decreasing the release of cytochrome-C from mitochondria (42). Previous studies declared that minocycline indirectly regulated the increase of the anti-apoptotic factors, e.g. BCL2 (43) and also inhibited the apoptosis proteins (44). Minocycline inhibits the activation of microglia and reduces the expression of the inflammatory mediators (45). It has been shown that minocycline is effective in chronic disorders caused by diabetes, and three week treatment with minocycline improved the diabetic neuropathy (46). It is important to state that long-term treatment with minocycline is completely safe (47). Compared with normal rats and sciatic nerve injury rats, TNF-α upregulation was in nerve cells and detectable at the damage site in the standard model of NP (48). It has been shown that the levels of TNF-α increased in the locus coeruleus and hippocampus (49). Upregulation of proinflammatory cytokines such as IL-1B, has been reported in the spinal cord and ipsilateral ganglia following nerve injury (50). In addition, it was reported that in the animal models of NP, the receptors of IL-1β have been induced by TNF-α receptor 1 (51). In the present study, minocycline reduced the levels of TNF-α and IL-1β, which may have partial roles in the efficacy of this agent in prevention of NP. Elevated levels of ROS induce persistent pain and related pain behaviors and also, it has been detected in ipsilateral superficial laminae (I-III) of the spinal cord (52). Preclinical evidence supports the fact that oxidative stress could enhance NP and induced hyperalgesia in the injured peripheral nerve and/or spinal cord (53). Our findings suggest that minocycline could be at least in part through the modulation of oxidative stress mitigated NP in the CCI model.

Conclusion

The present findings support that minocycline possessed anti-inflammatory and antioxidant effects in the chronic constriction injury induced NP and could prevent and at least delay the development of hypersensitivity in the CCI model.

Acknowledgment

Conflict of interest

The authors declare no conflicts of interest.

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