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Post-injury repeated administrations of minocycline improve the antinociceptive effect of morphine in chronic constriction injury model of neuropathic pain in rat

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ABSTRACT

It is confirmed that pharmacological attenuation of glial cells can alleviate neuropathic pain by lowering proinflammatory cytokine expression. The present study tries to confirm that post-injury administration of glia inhibitor, minocycline, can attenuate the neuropathic pain symptoms and improves the efficacy of morphine anti-nociception in chronic constriction injury (CCI). Male Wistar rats (230–270 g) underwent surgery for induction CCI model of neuropathy. For assessment of the thermal hyperalgesia and mechanical allodynia after CCI induction, morphine (2.5, 5, 7.5, 10 and 15 mg/kg; s.c.) and saline were administered on post-operative days (PODs) 0, 6 and 14. Hargreaves and Von–Frey tests were performed before and 30 min after morphine administration, respectively. The results showed significant decrease in antinociceptive effect of morphine on POD 6 compared to POD 0 only at the dose of 5 mg/kg. On the other hand, on POD 14 the antinociceptive effect of morphine (5, 7.5, 10 and 15 mg/kg) significantly decreased in comparison with POD 0. In another set of experiments, animals received minocycline (10, 20 and 40 mg/kg; i.p.) for eight days from POD 6 to 13 and then the antinociceptive effect of single dose of morphine 5 mg/kg was tested on POD 14. Behavioral tests showed that minocycline (40 mg/kg) could effectively attenuate the thermal hyperalgesia and mechanical allodynia on POD 13. Moreover, minocycline (40, 20 mg/kg) improved the anti-hyperalgesic, and minocycline (40 mg/kg) improved the anti-allodynic effects of morphine 5 mg/kg on POD 14. It seems that the reduction of antinociceptive effect of morphine after CCI may be mediated through glia activation. Modulation of glial activity by minocycline can attenuate CCI-induced neuropathic pain. It is also shown that repeated post-injury administration of minocycline improves the antinociceptive effect of morphine in neuropathic pain.

1. Introduction

Neuropathic pain, or chronic pain due to nerve injury, affects millions of people worldwide (Tsuda et al., 2005). It is a prevalent condition, for which currently there is no effective treatment (Zimmermann, 2001). Neuropathic pain is associated with severe chronic sensory disturbances characterized by spontaneous pain, increased responsiveness to painful stimuli (hyperalgesia), and pain perceived in response to normally non-noxious stimuli (allodynia). Pain associated with neuropathy is difficult to treat, often refractory to opioids, or requires high doses that possess unacceptable side effects (Hama and Borsook, 2005; Martin and Eisenach, 2001; Pascual et al., 2010). Recent evidence suggests that microglia and astrocytes activation plays an important role in initiation and maintenance of neuropathic pain (Watkins et al., 1997). Therefore, inhibiting glial cell activation could be a potential strategy to alleviate neuropathic pain.

Many studies indicate that neuropathic pain leads to reduced morphine efficacy and rapid development of morphine tolerance (Mayer et al., 1999; Mika et al., 2007). It has been hypothesized that neuropathic pain and morphine tolerance share some common cellular and molecular mechanisms (Mayer et al., 1999; Zhuo et al., 2011). It has been speculated that uncontrolled activation of microglia leads to altered activity of opioid systems or opioid-specific signaling (De la O-Arciniega et al., 2009; Speth et al., 2002; Watkins et al., 2007). The impairment of opioidergic transmission can be the reason of reduction of analgesic efficacy of morphine after nerve injury as a consequence of decreased number of presynaptic opioid receptors induced by loss of neurons (Ossipov et al., 1995; Porreca et al., 1998). It has been suggested that the suppression of glial activation, which in turn, inhibits proinflammatory cytokine synthesis, can improve morphine efficacy in treating neuropathic pain (Raghavendra et al., 2002; Song and Zhao, 2001; Watkins et al., 2007).

Minocycline, a semisynthetic second-generation tetracycline, with adequate penetration into the brain and cerebrospinal fluid (Aronson,

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1980; Colovic and Caccia, 2003) has emerged as a potential inhibitor of microglial activation and proliferation, without any known direct action on astrocytes or neurons (Amin et al., 1996; Tikka and Koistinaho, 2001). Recent reports indicate that intraperitoneal or intrathecal administration of minocycline could relieve neuropathic pain induced by nerve injury or peripheral inflammation (Guasti et al., 2009; Ledeboer et al., 2005; Raghavendra et al., 2003). These effects depend on inhibition of spinal microglia activation and proliferation, which consequently lower expression of proinflammatory cytokines. Studies indicate that intraperitoneal or intrathecal minocycline could exert anti-allodynic and anti-hyperalgesic effects on neuropathic pain when given preemptively (Guasti et al., 2009; Padi and Kulkarni, 2008). However, these results are difficult to be translated into clinical practice, because most patients with neuropathic pain seek treatment after signs of pain have occurred. Therefore, it is clear that post-injury treatment is more clinically relevant strategy. Some studies have suggested that minocycline administered early post-injury is useful for treating neuropathic pain (Ledeboer et al., 2005; Mei et al., 2011; Owolabi and Saab, 2006). However, the effect of repeated post-injury administration of minocycline on the treatment of nerve injury-induced neuropathic pain, and the efficacy of morphine at extended post-injury times is still unclear. The present study has evaluated the post-injury repeated administration of minocycline on chronic constriction injury induced neuropathic pain. Moreover, it was interesting to examine a possible influence of post-injury repeated minocycline administration on effectiveness of morphine in a rat model of neuropathic pain.

2. Materials and methods

2.1. Animals

Male Wistar rats (Pasture Institute, Tehran, Iran) weighing 230 ± 30 g (at the time of surgery) were used in this study. All animals were housed under standardized conditions in a room on a 12 h light/dark cycle with food and water available *ad libitum*. All experimental procedures followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals (Zimmermann, 1983) and were also approved by the research and ethics committee of Shahid Beheshti University of Medical Sciences. All tests were performed by the same person during the light phase.

2.2. Drugs

Morphine sulfate (Temad, Tehran, Iran) as an analgesic agent and minocycline (Sigma-Aldrich, Germany) as a microglia activation inhibitor, were dissolved in saline solution (0.9% NaCl) and injected in a volume of 2 ml/kg body weight.

2.3. Surgical operation

The chronic constriction injury (CCI) of the sciatic nerve was performed according to the method described by Bennett and Xie (Bennett and Xie, 1988). Briefly, rats were anesthetized by an intraperitoneal (i.p.) injection of a ketamine/xylazine mixture (60/7.5 mg/kg), and the sciatic nerve was exposed at the mid-thigh by dissection through the biceps femoris, proximal to the sciatic trifurcation, the nerve was exposed and four ligatures with chromic catgut 4–0 thread were tied loosely around the nerve with 1 mm space until a brief twitch was observed in the respective hind limb, the muscle and skin were closed in layers using 4–0 silk thread In sham-operated controls, an identical surgical procedure was performed, except the sciatic nerve was not ligated. All surgical procedures were performed under normal disinfected conditions by the same person. After surgery, we have used the Hargreaves test to confirm the successful induction of neuropathy before drugs or vehicle administration, according to Fig. 1A, a decrease of more than forty percent in PWL on POD 6 in comparison to POD 0 was considered as a cut-off point for the induction of neuropathic pain in operated hid paw.

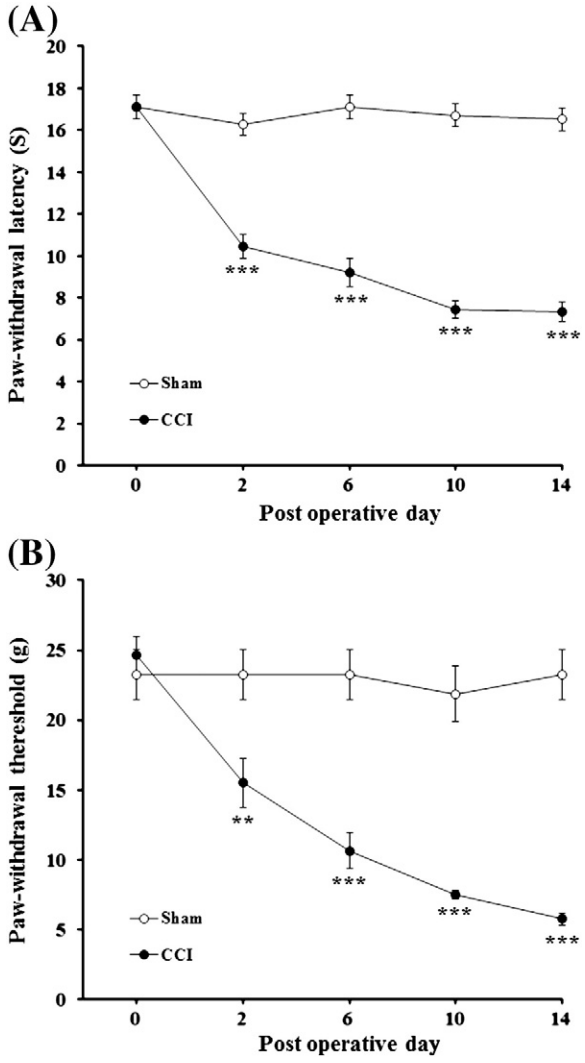

Fig. 1. Development of thermal hyperalgesia and mechanical allodynia after chronic constriction ligation of sciatic nerve in rat. (A) Paw withdrawal latency (S) measured by noxious radiant heat on different post-injury days. (B) Paw withdrawal threshold (gram) measured by the application of a series of Von–Frey filament. The data were presented a mean ± SEM (8 rats per group). Inter-group differences were analyzed by ANOVA Bonferroni's multiple comparison tests. **P<0.01 and ***P<0.001 indicate a significant difference between CCI and sham operated rats.

2.4. Behavioral tests

2.4.1. Thermal hyperalgesia

Thermal hyperalgesia was assessed by means of Hargreaves test of paw withdrawal latency (PWL). All tests were carried out between 8 and 12 am. The rats were placed in a clear plastic container on an elevated floor of clear, heat-tempered glass (Plantar Test, Ugo Basile, Italy). After 15 min period of habituation, a radiant heat source (50 W halogen reflector bulbs with intensity controlled by a constant voltage source) was focused on the plantar surface of the ipsilateral and contralateral hind paw. Each paw was tested five times at 5 min intervals and the average value of the withdrawal latency of five consecutive tests was recorded. The cut-off time in the absence of a response was 33 s to avoid tissue damage.

2.4.2. Tactile allodynia

The Von–Frey test was performed to assess the development of mechanical allodynia; all tests were carried out between 8 and 12 am. Rats were placed in transparent plastic experimental cages with wire mesh grid floors, after 15 min period of habituation, mechanical sensitivity of the ipsilateral and contralateral hind paw was assessed by measuring the paw withdrawal threshold (PWT), at which foot withdrawal to normally innocuous mechanical punctate stimuli were observed. Stimuli were delivered, from below to the plantar surface of the foot using 2, 4, 6, 8, 15, 26 and 60 g Von–Frey hair stimuli for a maximum of 5 s per application. Each trial consisted of the application of a series of Von–Frey hair, starting with an 8 g stimulus, and either increasing or decreasing in intensity until the PWT was observed. The paw withdrawal threshold was recorded as the lowest Von–Frey stimulus to elicit a response. The cut-off point in the absence of a response was 60 g to avoid tissue damage.

2.5. Experimental design

2.5.1. Dose–response curve of morphine after CCI induced neuropathic pain in rat

In these experiments, a dose–response relationship for antinociceptive effect of morphine after CCI-induced neuropathic pain in rat was established. In 5 groups (n = 8) of CCI animals, different doses of morphine (2.5, 5, 7.5, 10 and 15 mg/kg; s.c.) were administered one day before CCI, and then on days 6 and 14 after CCI. Vehicle-treated CCI animals (n = 8) received saline (2 ml/kg; s.c.) in the same manner as a control group. For evaluation of the changes in antinociceptive effect of morphine, thermal hyperalgesia and mechanical allodynia were assessed by Hargreaves and Von–Frey tests before and after each morphine administration, respectively. Finally, the morphine dose which showed more reduction in antinociceptive effect was selected for the next experiments. Data from the Hargreaves and Von–Frey tests were transformed as the percent of maximal possible effect (%MPE) by the following equation (Carmody, 1995):

$$\text{MPE\%} = \frac{\text{post} - \text{drug latency} - \text{pre} - \text{drug latency}}{\text{Cut off} - \text{pre} - \text{drug latency}} \times 100.$$

2.5.2. Post-injury administration of minocycline

To evaluate the effect of post-injury administration of minocycline on CCI-induced neuropathic pain, different doses of minocycline (10, 20 and 40 mg/kg; i.p.) administered daily from post-operative day (POD) 6 to 13 in CCI animals (n = 8). Vehicle-treated CCI animals (n = 8) received saline in the same manner as a control group. Sixty minutes after administration of the last doses of minocycline or vehicle on POD 13, the behavioral tests were performed. In addition, to study the improvement effect of minocycline on morphine-induced antinociception after CCI, different doses of minocycline (10, 20 and 40 mg/kg; i.p.) were injected daily from day 6 to 13 in CCI animals (n = 8). Vehicle-treated CCI animals (n = 8) received saline in the same manner as a control group. Finally on POD 14, a single dose of morphine (5 mg/kg; s.c.) was injected to all of these groups and 30 min later, the behavioral tests were examined.

2.6. Statistics

Data expressed as mean ± SEM (standard error of mean) and GraphPad Prism 5.0 (GraphPad Prism Software Inc., San Diego, CA, USA) was used for statistical analysis. In order to compare responses obtained from CCI and sham groups and also data from experimental groups of different morphine doses, two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used. Data from minocycline-treated and control groups were compared by one-way ANOVA, followed by Tukey's multiple comparison test. P-values less than 0.05 were considered to be statistically significant.

3. Results

Two-way repeated measures ANOVA revealed that the mean of paw-withdrawal latency (PWL) to thermal noxious stimuli [time effect: $F(4, 84) = 101.4$, $P<0.0001$; treatment effect: $F(2, 84) = 392.8$, $P<0.0001$; time × treatment effects: $F(8, 84) = 22.81$, $P<0.0001$; Fig. 1A] and paw-withdrawal threshold (PWT) to mechanical non-noxious stimuli [time effect: $F(4, 84) = 47.22$, $P<0.0001$; treatment effect: $F(2, 84) = 16.5$, $P<0.0001$; time × treatment: $F(8, 84) = 11.26$, $P<0.0001$; Fig. 1B] in CCI rats were significantly decreased compared to those in the sham-operated group of animals from POD 2 to 14 after nerve ligation. The thermal hyperalgesia and mechanical allodynia increased and reached steady state between POD 6 to 14 after nerve ligation (Fig. 1A,B). In order to study the development of tolerance to the antinociceptive effect of morphine in neuropathic pain, the anti-hyperalgesic and anti-allodynic effects of different doses of morphine were measured on days 0, 6 and 14 after nerve ligation.

3.1. Dose–response curve of morphine in CCI-induced neuropathic pain

The cumulative dose–response curve of morphine (2.5, 5, 7.5, 10 and 15 mg/kg; s.c.) on days 0, 6 and 14 after nerve ligation shifted to the right, which means that the anti-hyperalgesic [time effect: $F(2, 70) = 20.86$, $P<0.0001$; treatment effect: $F(4, 70) = 48.1$, $P<0.0001$; time × treatment: $F(8, 70) = 4.03$, $P = 0.042$; Fig. 2A] and anti-allodynic effects [time effect: $F(2, 70) = 10.16$, $P = 0.0002$; treatment effect: $F(4, 70) = 31.94$, $P<0.0001$; time × treatment: $F(8, 70) = 2.06$, $P = 0.75$; Fig. 2B] of morphine decreased after nerve ligation. It was also shown that on POD 6, low doses of morphine (2.5 and 5 mg/kg) failed to produce 50% of its maximal possible effect (MPE) in both tests. However, the antinociceptive effect of morphine 5 mg/kg showed more decrease on day 6 in comparison with day 0.

Nonetheless, on POD 14, morphine (2.5, 5 and 7.5 mg/kg) failed to show 50% of MPE in both tests. On POD 14, increasing the doses of morphine to 10 and 15 mg/kg retained its antinociceptive effect above the 50% of MPE. Since the dose of 5 mg/kg morphine shows more decrease in antinociceptive effect after CCI (from POD 0 to 14), this dose was selected to study the effect of repeated post-injury administration of minocycline in improving the analgesic effect of morphine.

3.2. Effects of chronic administration of minocycline on CCI-induced neuropathic pain

The effect of post-injury repeated administration of minocycline (10, 20 and 40 mg/kg; i.p.) and saline from POD 6 to 13 on thermal hyperalgesia and mechanical allodynia were evaluated by Hargreaves and Von–Frey tests in CCI animals. One-way ANOVA indicated significant difference between groups for PWL [$F(3, 28) = 8.76$, $P = 0.0003$; Fig. 3A] and PWT [$F(3,28) = 8.87$, $P = 0.0003$, Fig. 3B]. A Bonferroni post-hoc test revealed that the PWL in the group that received 40 mg/kg minocycline (12.75 ± 0.6 S; $P<0.001$) was significantly higher than that in control group (8.7 ± 0.31 S). Moreover, PWT in groups that received 20 mg/kg (17.75 ± 1.8 g; $P<0.01$) and 40 mg/kg (20.5 ± 2.07 g; $P<0.001$) minocycline were significantly higher than that in control group (9.25 ± 1.29 g) as well.

3.3. Effects of chronic administration of minocycline on antinociceptive effect of morphine in CCI-induced neuropathic pain

To evaluate the effects of minocycline on anti-hyperalgesic and anti-allodynic effects of morphine (5 mg/kg; s.c.) in neuropathic pain, minocycline (10, 20 and 40 mg/kg; i.p.) and saline were administered

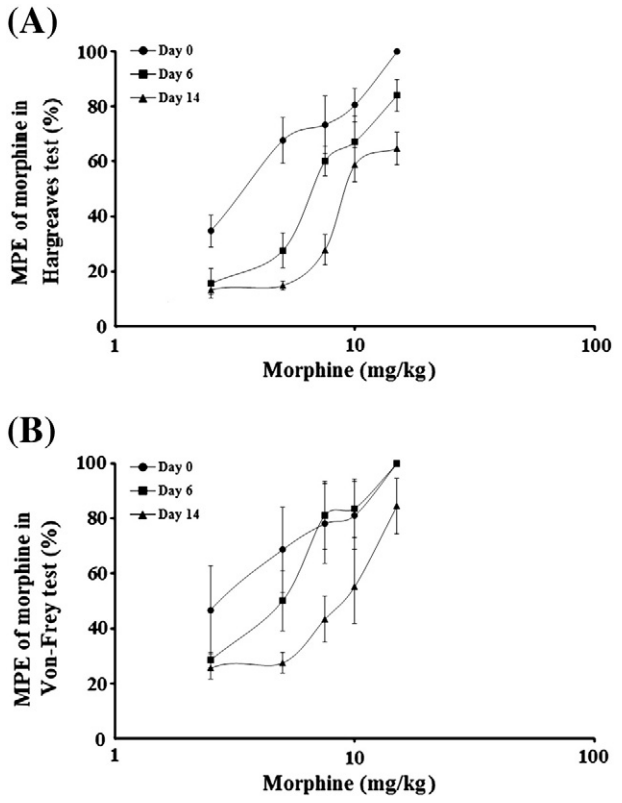

Fig. 2. Cumulative dose–response curve of morphine (2.5, 5, 7.5, 10 and 15 mg/kg; s.c.) on days 0, 6 and 14 after surgery. (A) Anti-hyperalgesic and (B) anti-allodynic effects of morphine significantly decreased after induction of CCI neuropathy in rat as shown by rightward shift dose–response curve. Hyperalgesia and allodynia were assessed 30 min after morphine injection. Data were calculated as the percentage of maximal possible effect (%MPE) that represents as mean ± SEM (8 rats per group).

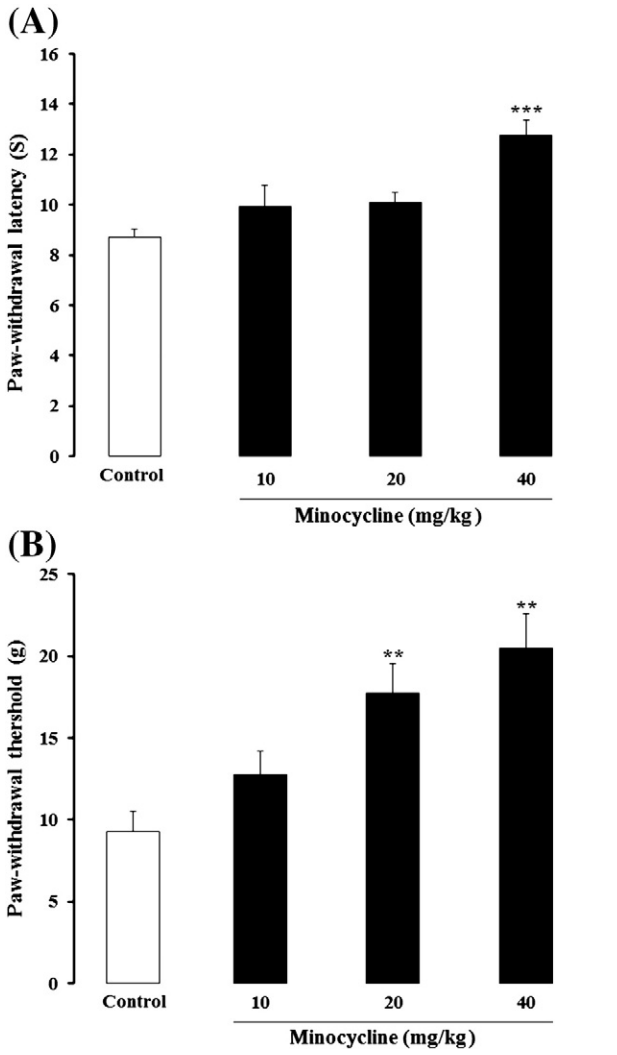

Fig. 3. Influence of post-injury repeated administration of minocycline (10, 20 or 40 mg/kg; i.p.) from POD 6 to POD 13 on the development of (A) thermal hyperalgesia and (B) mechanical allodynia, after CCI in rats. Hyperalgesia and allodynia were assessed 60 min after last doses of minocycline administration. In control group, CCI animal received saline instead of minocycline. The data are presented as mean ± SEM (8 rats per group). Inter-group differences were analyzed by ANOVA Bonferroni's multiple comparison tests. **P<0.01 and ***P<0.001 indicate a significant difference compared with control group.

from POD 6 to 13 in CCI animals, and one day later on POD 14 the Hargreaves and Von–Frey tests were examined 30 min after morphine 5 mg/kg injection. One-way ANOVA indicated significant difference between groups for anti-hyperalgesic [$F(3, 28) = 6.52$, $P = 0.0017$; Fig. 4A] and anti-allodynic [$F(3, 28) = 10.44$, $P < 0.0001$; Fig. 4B] effects of morphine. In groups that animals received 20 mg/kg (21.3 ± 1.66 S; $P < 0.05$) and 40 mg/kg (24.06 ± 2.17 S; $P < 0.01$) minocycline, the anti-hyperalgesic effect of morphine (PWL) was significantly higher than that in control group (14.19 ± 0.72 S). Additionally, anti-allodynic effect of morphine (PWT) in the group that received minocycline 40 mg/kg (51.5 ± 5.56 g; $P < 0.001$) was significantly higher than that in control group (21.88 ± 2.01 g).

3.4. The maximal possible effect of morphine in CCI-induced neuropathic pain after chronic administration of minocycline

In order to compare the difference in PWT and PWL before and after morphine administration in all groups, we calculated the %MPE of morphine in all experimental groups by the following formula:

$$\%\text{MPE} = \frac{\text{Response of POD 14} - \text{Response of POD 13}}{\text{Cut off} - \text{Response of POD 13}}.$$

One-way ANOVA indicated significant difference between groups for anti-hyperalgesic [$F(3, 28) = 4.3$, $P = 0.012$; Fig. 4C] and anti-allodynic [$F(3, 28) = 7.73$, $P < 0.005$; Fig. 4D] effects of morphine. In animals that received minocycline 20 mg/kg (48.58 ± 7.62%; $P < 0.05$) and 40 mg/kg (55.73 ± 10.77%; $P < 0.01$), mean %MPE of morphine in Hargreaves test were significantly higher than those in control group (19.85 ± 2.08%). Additionally, in animals that received minocycline 40 mg/kg (81.11 ± 12.37%; $P < 0.001$), the mean %MPE of morphine in Von–Frey test was significantly higher than that in control group (24.75 ± 3.73%).

4. Discussion

It has been reported that CCI model mimics some characteristics of neuropathic pain in humans (Bennett and Xie, 1988); CCI can induce rapid and significant mechanical allodynia and thermal hyperalgesia in rat (Dowdall et al., 2005; Mika et al., 2007), which was supported in this study. In addition, we observed that repeated post-injury administration of minocycline attenuated the development of allodynia and hyperalgesia in rat after CCI. In this study, it has been also shown that post-injury repetitive administration of minocycline improves the antinociceptive effect of morphine after CCI-induced neuropathic pain. Spinal microglia activation is thought to be a key factor in initiation of CCI-induced neuropathic pain, and inhibition of spinal microglia activation could be an efficient way for treating neuropathic pain (Mika et al., 2007; Raghavendra et al., 2003; Tsuda et al., 2005). It seems that the activated glial cells in the spinal cord release proinflammatory cytokines and other substances that facilitate pain transmission (Coyle,

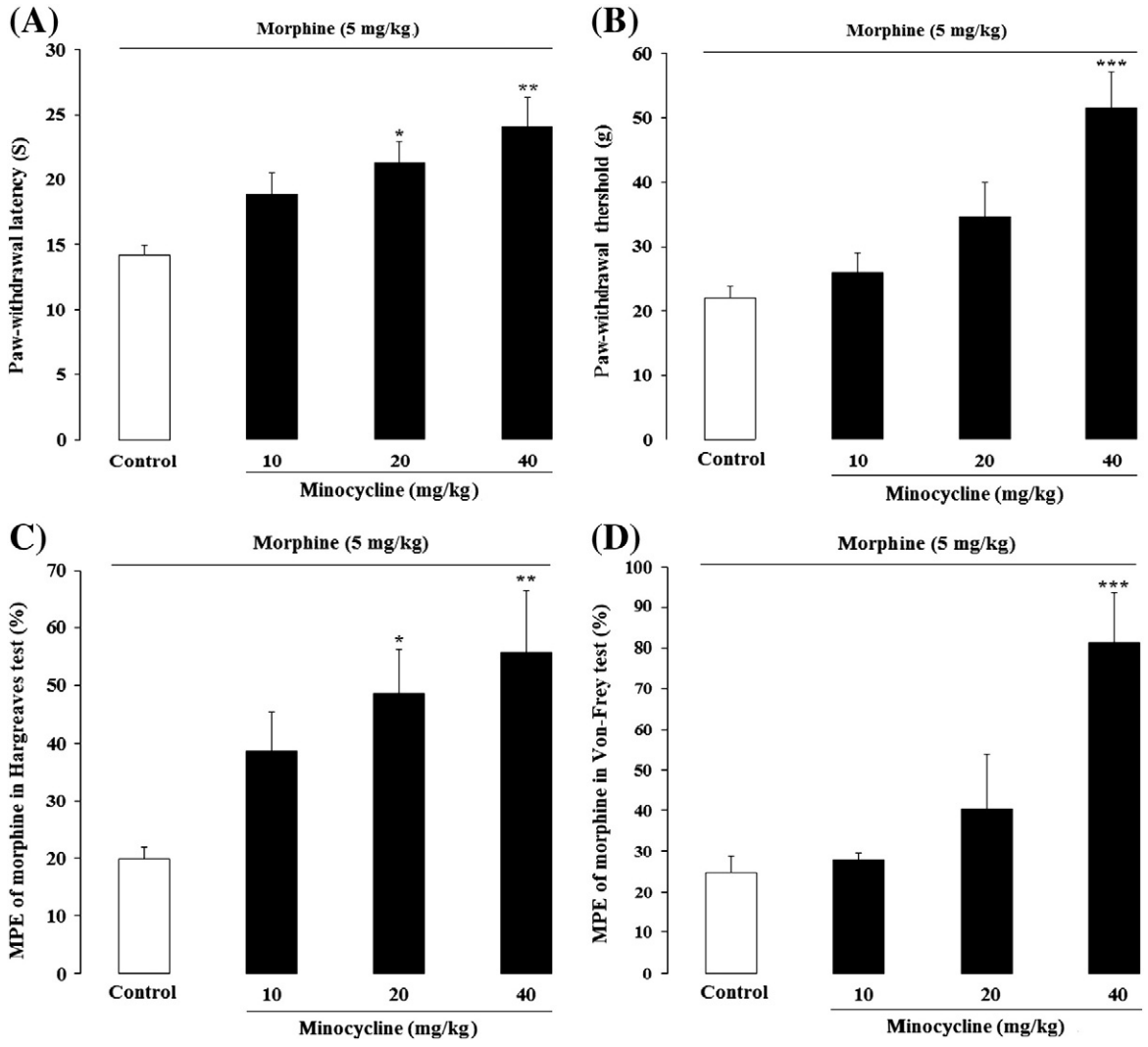

Fig. 4. Influence of post-injury repeated administration of minocycline (10, 20 or 40 mg/kg; i.p.; from POD 6 to POD 13) on (A) anti-hyperalgesic and (B) anti-allodynic effects of a single dose of morphine (5 mg/kg; s.c.), on day 14 after CCI in rats. In control group, CCI animal received saline instead of minocycline and then a single dose of morphine on POD 14. Hyperalgesia and allodynia were assessed 30 min after morphine injection. Also, in minocycline and saline treated groups, we calculated the percentage of maximal possible effect (%MPE) of a single dose of morphine (5 mg/kg; s.c.), in (C) Hargreaves test and (D) Von–Frey test. The difference in PWL and PWT before (POD 13) and after (POD 14) morphine administration was calculated as %MPE of morphine. The data were presented as mean ± SEM (8 rats per group). Inter-group differences were analyzed by ANOVA Bonferroni's multiple comparison tests. *P<0.05, **P<0.01 and ***P<0.001 indicate a significant difference compared with control (vehicle-treated CCI rats which received a single dose of morphine).

1998; Watkins and Maier, 2003). It is reported that minocycline reduces microglial activity by inhibiting p38 mitogen-activated protein kinase (MAPK) in microglia (Piao et al., 2006). Previous studies have shown that pre-emptive administration of minocycline prevents neuropathic pain that is induced by peripheral inflammation or nerve injury by inhibition of microglia activation (Mika et al., 2010). On the other hand, recent findings have indicated that post-injury administration of single intrathecal minocycline is an effective way to treat spinal nerve ligation (SNL)-induced neuropathic pain, and the therapeutic time window for minocycline to alleviate mechanical allodynia is found only on POD 3 and POD 7, but not on POD 10 or POD 21 (Mei et al., 2011). Another study showed that post-injury treatment with ropivacaine, a local anesthetic drug, in sub-anesthetic dose that inhibits glia activation caused the attenuation of thermal hyperalgesia in CCI-induced neuropathic rat (Toda et al., 2011). In our study, we showed that chronic administration of minocycline from POD 6 to 13 could attenuate CCI-induced mechanical allodynia and thermal hyperalgesia.

Nonetheless, the key point of this study was to clarify that post-injury intraperitoneal minocycline could be an effective way for improvement of the antinociceptive effect of morphine in treating neuropathic pain. We have shown that the antinociceptive effect of morphine reduces during development of neuropathic pain in CCI. These results are concurrent with other studies which show the development of tolerance to antinociceptive effect of systemic morphine in SNL (Christensen et al., 2000; Raghavendra et al., 2002). However, we found that treatment of animal with minocycline from day 6 to 13 after induction of CCI significantly potentiates the anti-allodynic and anti-hyperalgesic effects of single dose of morphine (5 mg) that was measured on day 14 after CCI. Resistance to analgesic effect of morphine is characteristic of neuropathic pain (McQuay, 2002; Porreca et al., 1998). The mechanisms which decreased analgesic efficacy of morphine in neuropathic pain are not fully understood. It was suggested that lower effectiveness of morphine in neuropathic pain might be due to the reduced number of presynaptic opioid receptors following degeneration of primary afferent neurons after nerve damage (Porreca et al., 1998). It is well understood that neuropathic pain and morphine tolerance share some common cellular and molecular mechanisms (Mayer et al., 1999), and activation of glia is a common point in pathophysiological changes of neuropathic pain and morphine tolerance (Song and Zhao, 2001). Although the role of glia and cytokines in the initiation and maintenance of pain states has been reported (Ledeboer et al., 2005; Watkins et al., 2005; Watkins and Maier,

2003), less information is available about influence of modulation of glial activity on analgesic effects of morphine in neuropathic pain. It has been suggested that activation of microglial cells and enhanced release of proinflammatory cytokines in spinal cord is responsible for the development of morphine tolerance after nerve injury (Raghavendra et al., 2002; Raghavendra et al., 2003; Raghavendra et al., 2004). It is already known that microglia release proinflammatory cytokines (IL-1, IL-6 and TNF alpha) in response to morphine, thereby opposing its effects (Johnston and Westbrook, 2005; Raghavendra et al., 2002; Raghavendra et al., 2003; Raghavendra et al., 2004).

In conclusion, this study showed that antinociceptive effect of morphine decreases during the development of neuropathic pain and use of agents that selectively inhibit microglial activity may maintain the beneficial effect of opioids in neuropathic pain. In addition, post-injury systemic administration of minocycline is an effective way to attenuate the CCI-induced neuropathic pain.

Acknowledgments

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