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ARTICLE

Anticonvulsant effect of minocycline on pentylenetetrazole-induced seizure in mice: involvement of nitric oxide and *N*-methyl-D-aspartate receptor

Hossein Amini-Khoei, Nastaran Kordjazy, Arvin Haj-Mirzaian, Shayan Amiri, Arya Haj-Mirzaian, Armin Shirzadian, Amin Hasanvand, Shima Balali-Dehkordi, Mahsa Hassanipour, and Ahmad Reza Dehpour

Abstract: Anticonvulsant effects of minocycline have been explored recently. This study was designed to examine the anticonvulsant effect of acute administration of minocycline on pentylenetetrazole-induced seizures in mouse considering the possible role of the nitric oxide/*N*-methyl-D-aspartate (NMDA) pathway. We induced seizure using intravenous administration of pentylenetetrazole. Our results showed that acute administration of minocycline increased the seizure threshold. Furthermore, co-administration of subeffective doses of the nonselective nitric oxide synthase (NOS) inhibitor N^G-L-arginine methyl ester (10 mg/kg) and the neuronal NOS inhibitor 7-nitroindazole (40 mg/kg) enhanced the anticonvulsant effect of subeffective doses of minocycline (40 mg/kg). We found that inducible NOS inhibitor aminoguanidine (100 mg/kg) had no effect on the antiseizure effect of minocycline. Moreover, L-arginine (60 mg/kg), as a NOS substrate, reduced the anticonvulsant effect of minocycline. We also demonstrated that pretreatment with the NMDA receptor antagonists ketamine (0.5 mg/kg) and MK-801 (0.05 mg/kg) increased the anticonvulsant effect of subeffective doses of minocycline. Results showed that minocycline significantly decreased the hippocampal nitrite level. Furthermore, co-administration of a neuronal NOS inhibitor like NMDA receptor antagonists augmented the effect of minocycline on the hippocampal nitrite level. In conclusion, we revealed that anticonvulsant effect of minocycline might be, at least in part, due to a decline in constitutive hippocampal nitric oxide activity as well as inhibition of NMDA receptors.

Key words: minocycline, anticonvulsant, nitric oxide, NO, neuronal nitric oxide synthase, nNOS, *N*-methyl-D-aspartate, NMDA.

Résumé : On a récemment exploré les effets anticonvulsivants de la minocycline. Cette étude a été conçue en vue d'examiner l'effet anticonvulsivant de l'administration aiguë de minocycline sur des convulsions provoquées par le pentylènetétrazole chez la souris, en tenant compte du rôle éventuel de la voie de signalisation oxyde nitrique/*N*-méthyl-D-aspartate (NMDA). Nous avons provoqué des convulsions par l'administration intraveineuse de pentylènetétrazole. Nos résultats ont montré que l'administration aiguë de minocycline augmentait le seuil des convulsions. En outre, l'administration concomitante de doses sous-efficaces de « N^G-L-arginine methyl ester » (inhibiteur non sélectif de l'oxyde nitrique synthase (NOS); 10 mg/kg) et de 7-nitroindazole (inhibiteur de la NOS neuronale; 40 mg/kg) entraînait une augmentation de l'effet anticonvulsivant de doses sous efficaces de minocycline (40 mg/kg). Nous avons observé que l'aminoguanidine (inhibiteur de la NOS inductible; 100 mg/kg) n'avait aucun effet sur les effets anticonvulsivants de la minocycline. De surcroît, la L-arginine (60 mg/kg), en tant que substrat de la NOS, entraînait une réduction de l'effet anticonvulsivant de la minocycline. Nous avons aussi montré que l'administration préalable d'antagonistes des récepteurs du NMDA, de kétamine (0,5 mg/kg) et de MK-801 (0,05 mg/kg), entraînait une augmentation de l'effet anticonvulsivant de doses sous-efficaces de minocycline. Les résultats ont montré que la minocycline entraînait une diminution marquée des concentrations de nitrite dans l'hippocampe. En outre, l'administration concomitante d'inhibiteurs de la NOS neuronale comme des antagonistes des récepteurs du NMDA entraînait une augmentation de l'effet de la minocycline sur les taux de nitrite dans l'hippocampe. En conclusion, nous avons révélé que l'effet anticonvulsivant de la minocycline pourrait être, au moins en partie, causé par la diminution de l'activité de l'oxyde nitrique constitutif dans l'hippocampe, ainsi que par l'inhibition des récepteurs du NMDA. [Traduit par la Rédaction]

Mots-clés : minocycline, anticonvulsivant, oxyde nitrique, NO, oxyde nitrique synthase neuronale, nNOS, *N*-méthyl-D-aspartate, NMDA.

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Introduction

Epilepsy arises due to abnormal and excessive neuronal activity of the brain and is recognized as a syndrome characterized by additional co-occurring conditions (Fisher et al. 2014). Regardless of the development of numerous antiepileptic drugs, new agents with further anticonvulsant activity and fewer side effects are interesting goals in neurology (Hitiris and Brodie 2006).

Minocycline, a second-generation tetracycline with broad-spectrum antimicrobial properties, has perfect bioavailability and outstanding blood–brain barrier permeability (Macdonald et al. 1973). Inhibition of oxidative stress (Morimoto et al. 2005) and nitric oxide synthase (NOS) (Kim et al. 2004), blockade of *N*-methyl-D-aspartate (NMDA) receptors (Munzar et al. 2002), and anti-inflammatory (Yrjänheikki et al. 1999) properties have been explained for neuroprotective effects of minocycline. Recently, the anti-inflammatory agents and scavengers have been explored by researchers (Jami et al. 2014, 2015). In this regard, evidence shows that anti-inflammatory drugs that inhibit activation of microglia possessed neuroprotective properties (Vezzani et al. 2011). There are several preclinical studies reporting the protective effects of minocycline on seizures (Wang et al. 2012; Wang et al. 2015; Barker-Haliski et al. 2016). Besides animal studies, Nowak and co-workers demonstrated that minocycline therapy significantly reduced the seizure frequency in patients (Nowak et al. 2012).

On the other hand, it is well known that the overactivation of the glutamatergic system plays a fundamental role in epilepsy (Urbańska et al. 1992). The NMDA receptor activity contributes to neuronal excitation and is linked to the pathophysiology of epilepsy (Ghasemi and Dehpour 2014). It is well determined that NMDA receptor blockers possess antiepileptic activity (Sato et al. 1988; Cotton et al. 1993; Freitas et al. 2006; Vataev et al. 2009). Furthermore, ample evidence suggests that anticonvulsant effects of antiepileptic drugs are mediated, in part, via blockade of NMDA receptors (Ghasemi et al. 2010; Rahimi et al. 2014).

Nitric oxide (NO) is a neuromodulator involved in different biological functions of the nervous system (Esplugues 2002; Sadaghiani et al. 2011). Activation of the NMDA receptors initiates production and release of NO in the brain (Diniz et al. 2016). It has been shown that NO contributes to the pathophysiology of seizures (Riazi et al. 2006). NOS produces NO from the precursor L-arginine (Ostadhadi et al. 2016). It has been shown that blockade of NOS is involved in the anticonvulsant effect of many drugs in animal models of seizure (Bagetta et al. 2002; Riazi et al. 2006; Bahremand et al. 2010*a*).

Although the contribution of NO in underlying mechanisms of minocycline has been reported previously, there is little evidence about the involvement of the NO pathway in the anticonvulsant effect of minocycline in an experimental model of pentylenetetrazole (PTZ) induced seizure. Moreover, according to the inhibitory effects of minocycline on neuroexcitation, the role of NMDA receptors in the antiseizure effect of minocycline gained our interest. Since little is known about the involvement of NMDA receptors and NO in the protective effect of minocycline, we firstly aimed to examine the anticonvulsant effect of acute administration of minocycline on PTZ-induced seizure. Secondly, we investigated the possible contribution of the NO pathway and NMDA receptors in this effect.

Materials and methods

Animals

Male NMRI mice, 20–30 g mass (Pasteur Institute of Iran, Tehran, Iran), were used in this study. Animals were housed in groups (four or five mice per cage) and kept at a temperature of 21–23 °C under a 12 h regular light–dark cycle and access to food and water ad lib. All experiments were carried out between 1200 and 1600. Each experimental group included six animals in behavioral experiments and four animals in biochemical tests and each mouse was used only once.

Drugs

The following drugs were used: minocycline, PTZ, N^G-L-arginine methyl ester (L-NAME), aminoguanidine, 7-nitroindazole (7-NI), L-arginine (L-arg), ketamine, and MK-801 (all purchased from Sigma, St. Louis, Missouri, USA). 7-NI was dissolved in a 1% solution of Tween 80 and all other drugs were freshly dissolved in physiological saline and were prepared immediately before the administrations. Except for PTZ, which was administered via the intravenous route, all injections were through the intraperitoneal (i.p.) route and with a volume of 5 mL/kg body mass.

Treatments

In the first step, we examined the effects of different doses of minocycline (20, 40, 80, and 120 mg/kg) on seizure threshold. Control animals in this step received acute i.p. injections of the appropriate vehicle (saline at 5 mL/kg). In the next step, minocycline (120 mg/kg) was administered at 30, 60, and 120 min prior to PTZ injection.

We assessed the effects of L-NAME (a nonselective NOS inhibitor, 10 mg/kg) (Amiri et al. 2016*b*; Haj-Mirzaian et al. 2016*a*), aminoguanidine (a selective inducible NOS (iNOS) inhibitor, 100 mg/kg) (Shafaroodi et al. 2012), 7-NI (a selective neuronal NOS (nNOS) inhibitor, 40 mg/kg) (Yahyavi-Firouz-Abadi et al. 2006), and L-arg (an NO precursor, 60 mg/kg) (Payandemehr et al. 2014) on the seizure threshold in mice. L-NAME and aminoguanidine were administered 60 min, L-arginine 45 min, and 7-NI 30 min before administration of PTZ (Yahyavi-Firouz-Abadi et al. 2006). Furthermore, the possible involvement of the nitrergic system on the anticonvulsant activity of minocycline was examined through co-administration of the subeffective doses of L-NAME (simultaneous administration with minocycline), aminoguanidine (simultaneous administration with minocycline), and 7-NI (30 min after minocycline administration) with the subeffective dose of minocycline. Moreover, the effective dose of minocycline was co-administered with the effective dose of L-arg (15 min after minocycline administration). To exclude the effect of vehicle administration on behavioral assessments, saline or 1% solution of Tween 80 (5 mL/kg) was injected before PTZ into the control groups.

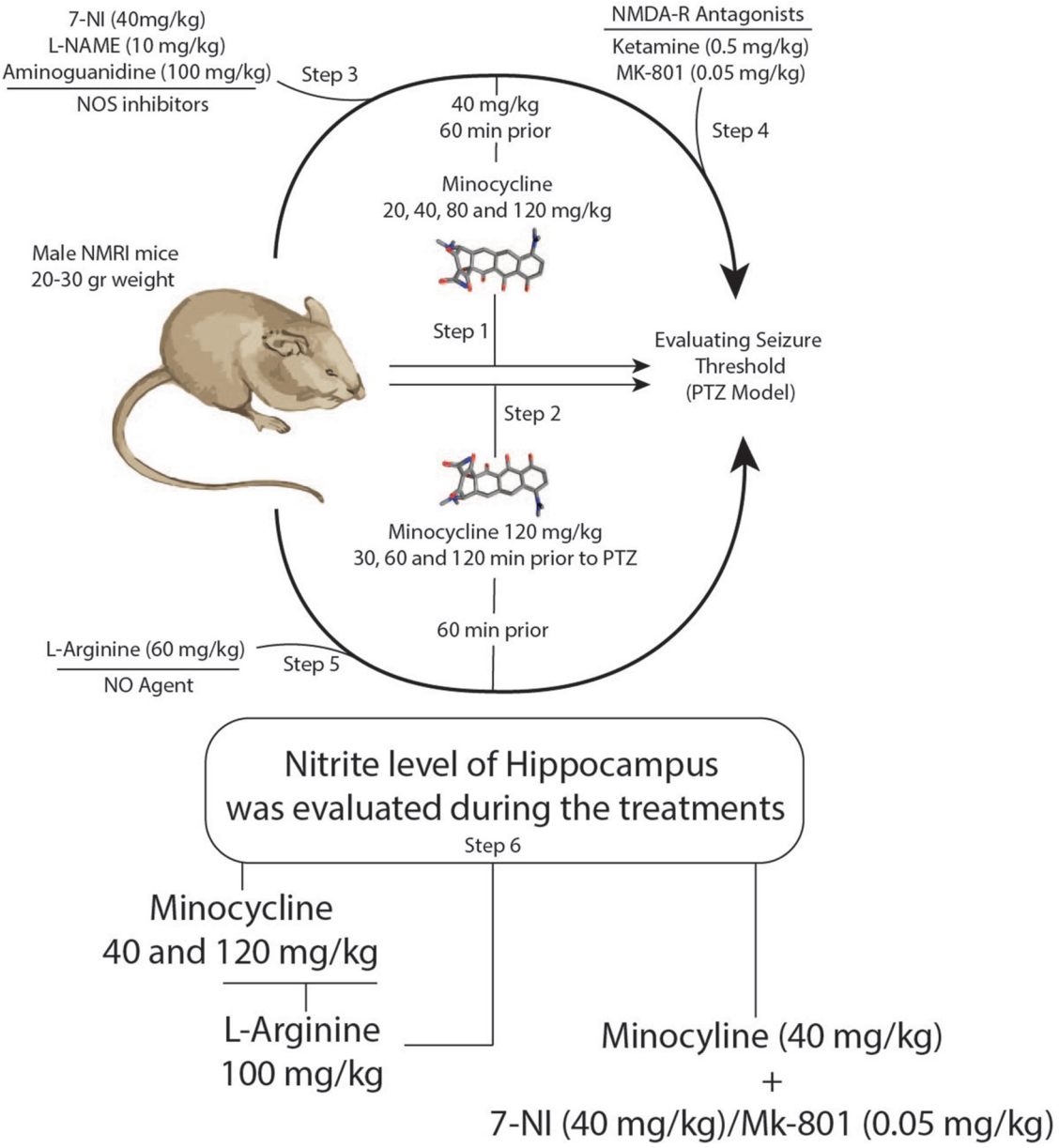
In the next part, we examined the effects of ketamine (0.5 mg/kg) and MK-801 (0.05 mg/kg) on the seizure threshold (Ghasemi et al. 2010; Amiri et al. 2016*a*). Ketamine and MK-801 were administered 60 min before PTZ injection. Furthermore, the probable involvement of NMDA receptors in the anticonvulsant activity of minocycline was examined through co-administration of the subeffective doses of ketamine and MK-801 with the subeffective dose of minocycline. To rule out the effect of vehicle administration on behavioral assessments, saline (5 mL/kg) was injected 60 min before PTZ into the control groups. The doses and times of administrations were chosen based on our pilot study and also on previously published studies (Homayoun et al. 2002*b*; Yahyavi-Firouz-Abadi et al. 2006; Ghasemi et al. 2010; Rahimi et al. 2014; Amiri et al. 2016*a*). Figure 1 shows the chronologic design of this study.

Further, we measured the hippocampal nitrite levels following treatment with minocycline (40 and 120 mg/kg) and also subsequent co-administration of minocycline with NO/NMDA agents (L-arg, 7-NI, and MK-801). After these treatments (60 min after minocycline injection), animals were euthanized under anesthesia using diethyl ether and hippocampi were dissected on an ice-cold surface and immediately immersed in liquid nitrogen. Tissue homogenates were prepared and hippocampal nitrite content was measure using the described method.

Determination of clonic seizure threshold (CST)

To determine the CST in mice, we used the previously described method (Amiri et al. 2014, 2016*b*, 2017; Amini-Khoei et al. 2015).

Fig. 1. Chronologic study design. [Colour online.]



Briefly, a winged infusion set (30 gauge) was used to infuse the PTZ (0.5%) at a constant rate of 1 mL/min into the tail vein of the freely moving subject. We stopped the infusion whenever forelimb clonus followed by full clonus of the body occurred (began with running and then loss of righting ability). The minimal dose of PTZ (milligrams per kilogram of mouse mass) needed to induce a clonic seizure was considered as the index of seizure threshold. Because PTZ was administrated at a constant rate (1 mL/min), duration to induce seizure depended on dose and time of PTZ injection (Amiri et al. 2016*a*).

Nitrite assay

To determine the NO level in the hippocampus, we measured the nitrite level as the result of the NO end product (Ding et al. 2010; Kordjazy et al. 2015; Haj-Mirzaian et al. 2016*b*). Nitrite level was measured with a colorimetric assay based on the Griess reaction in each hippocampus sample. Concentration of nitrite was determined by reference to a standard curve of sodium nitrite (Sigma) and normalized to the mass of each sample.

Ethics declarations

The protocol for this project has been approved by the ethics committee of the Tehran University of Medical Sciences (Tehran, Iran) and all experiments were performed according to the institutional guidelines for animal care and use. All animal manipulations were carried out according to the "Guide for the care and use of laboratory animals (8th ed., 2011, published by the National Academies Press, Washington, DC, USA) and legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU).

Statistical analysis

Data were expressed as mean ± SEM of CST in each experimental group. One-way ANOVA followed by Tukey's post hoc comparison was used to analyze the results. The GraphPad Prism software (version 6) was used to illustrate the results. The analysis was based on normally distributed data and a P value of less than 0.05 was considered statistically significant. The sample size was calculated by power calculations using G power software (ver.3.1.7)

Fig. 2. Time course effect of minocycline on pentylenetetrazole (PTZ) induced seizure: effect of acute minocycline administration on clonic seizure threshold (CTS) in PTZ-injected mice. Minocycline (120 mg/kg) was injected 30, 60, and 120 min prior to PTZ injection and the CST was evaluated. Values are expressed as the mean ± SEM analyzed by one-way ANOVA followed by Dunnett's multiple comparison test (n = 6 for all groups). $^{*}P < 0.05$ and $^{***}P < 0.001$ versus the control group (t = 0). PTZ was administrated at a constant rate of 1 mL/min.

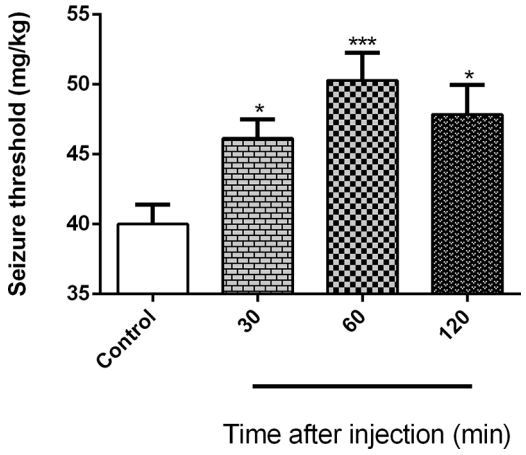
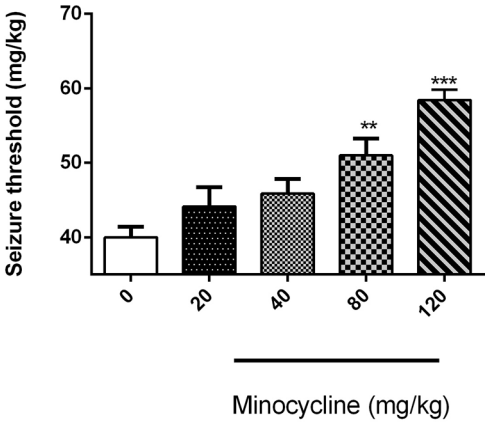


Fig. 3. Effect of different doses of minocycline on pentylenetetrazole (PTZ) induced seizure: effect of acute administration of minocycline (20, 40, 80, and 120 mg/kg i.p. 60 min before PTZ injection) on the clonic seizure threshold in PTZ-induced seizures in mice. Values are expressed as the mean ± SEM analyzed by one-way ANOVA followed by Dunnett's multiple comparison test (n = 6 for all groups). $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the control (saline-injected) group. PTZ was administrated at a constant rate of 1 mL/min.



(Franz Faul, Universitat Kiel, Germany). The α error was set at 0.05 and the power (1 – β) at 0.8 and the required total sample size per group was calculated as six to eight in behavioral tests and four in biochemical tests. Also, it should be noted that the post hoc power analysis of all statistical reports was more than 80%.

Results

Effect of minocycline on seizure threshold

Figure 2 shows the time course of the anticonvulsant effect of minocycline (120 mg/kg). The data showed that minocycline exerted an anticonvulsant effect ($F_{[3,25]}$ = 44.04, $P < 0.001$) with maximal effect at 60 min after administration ($P < 0.001$) in comparison with saline-treated animals.

Figure 3 shows the effects of acute i.p. administration of various doses of minocycline on PTZ-induced seizure. One-way ANOVA revealed a significant anticonvulsant effect for minocycline ($F_{[4,25]}$ = 10.12, $P < 0.001$) at doses of 80 mg/kg ($P < 0.05$) and 120 mg/kg ($P < 0.001$) with maximal effect at 120 mg/kg. Minocycline at doses of 20 and 40 mg/kg did not produce a significant anticonvulsant effect when compared with the control group ($P > 0.05$).

Effects of NOS inhibitors on the anticonvulsant effect of minocycline

We examined the effect of the nonselective NOS inhibitor L-NAME on the anticonvulsant effect of minocycline (Fig. 4A). Analysis with one-way ANOVA revealed a significant effect for L-NAME ($F_{[3,20]}$ = 11.68, $P < 0.001$). Tukey's post hoc comparison showed that L-NAME (10 mg/kg) did not alter the CST in the control groups. Nevertheless, L-NAME (10 mg/kg) enhanced the anticonvulsant effect induced by minocycline (40 mg/kg) compared with the control group ($P < 0.001$).

Figure 4B shows the effect of 7-NI on minocycline-induced modulation of seizure threshold. One-way ANOVA determined a significant effect for 7-NI ($F_{[3,20]}$ = 18.03, $P < 0.001$). Injection of 7-NI alone (40 mg/kg) did not change the CST but augmented the anticonvulsant effect of minocycline (40 mg/kg) compared with the control group ($P < 0.001$).

We studied the effect of the iNOS inhibitor aminoguanidine on the anticonvulsant effect of minocycline to test the possible involvement of iNOS. Analysis with one-way ANOVA did not show a significant effect for aminoguanidine ($F_{[3,20]}$ = 2.816, $P > 0.05$). Similarly, pretreatment with aminoguanidine (100 mg/kg) did not affect the CST in either saline or minocycline-treated (40 mg/kg) groups ($P > 0.05$) (Fig. 4C).

Effect of co-administration of L-arg with minocycline on CST

One-way ANOVA analysis revealed a significant effect for treatment with the NO precursor L-arg (60 mg/kg) on CST ($F_{[3,20]}$ = 10.42, $P < 0.001$) (Fig. 5). Post- hoc test evaluation showed that L-arg alone did not change the CST in control animals. However, L-arg in combination with minocycline significantly reversed the anticonvulsant effect of minocycline ($P < 0.01$ compared with the minocycline group).

Effects of NMDA antagonists on the anticonvulsant effect of minocycline

We examined the effect of NMDA antagonists on the anticonvulsant effect of minocycline (Fig. 6). One-way ANOVA showed a significant effect for ketamine ($F_{[3,20]}$ = 6.624, $P < 0.01$) (Fig. 6A) and MK-801 ($F_{[3,20]}$ = 8.843, $P < 0.01$) (Fig. 6B). Analysis with one-way ANOVA followed by Tukey's post hoc test demonstrated that ketamine (0.5 mg/kg) and MK-801 (0.05 mg/kg) did not alter the CST in the control (vehicle-received) groups, but both of them enhanced the anticonvulsant effect of minocycline (40 mg/kg) compared with either drug alone ($P < 0.01$).

Hippocampal nitrite assay

One-way ANOVA determined the significant effects of minocycline treatment on the hippocampal nitrite levels ($F_{[2,9]}$ = 19.60, $P < 0.001$) (Fig. 7A). Tukey's post hoc analysis revealed that minocycline (120 mg/kg) (but not 40 mg/kg) induced a significant decline in the hippocampal nitrite levels compared with saline-administered animals ($P < 0.01$).

In the next part, the possible effect of minocycline and nNOS inhibitor/NMDA receptor antagonist co-administration on the hippocampal nitrite level was evaluated ($F_{[5,18]}$ = 3.466, $P < 0.05$) (Fig. 7B). Results obtained from Tukey's post hoc test revealed that administration of 7-NI (40 mg/kg), MK-801 (0.05 mg/kg), and minocycline (40 mg/kg) alone failed to change the hippocampal nitrite content; however, co-administration of a subeffective dose of 7-NI with the subeffective dose of minocycline produced a significant

Fig. 4. Effect of pretreatment with nitric oxide synthase inhibitors on the anticonvulsant effect of minocycline: effect of administration of (A) N^G-L-arginine methyl ester (LMN) (10 mg/kg), (B) 7-nitroindazole (7-NI) (40 mg/kg), and (C) aminoguanidine (AG) (100 mg/kg) with saline or minocycline (Mino) (40 mg/kg) on the clonic seizure threshold in pentylenetetrazole (PTZ) injected mice. Values are expressed as the mean ± SEM analyzed by one-way ANOVA followed by Tukey's multiple comparison test (n = 6 for all groups). ***P < 0.001 versus the vehicle-injected group. PTZ was administrated at a constant rate of 1 mL/min.

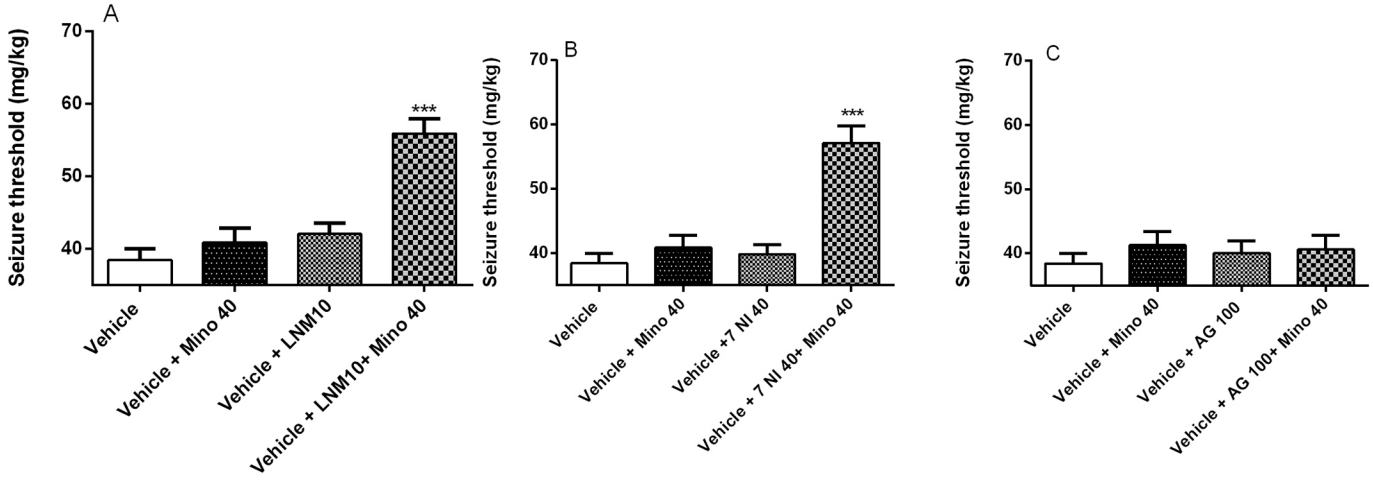
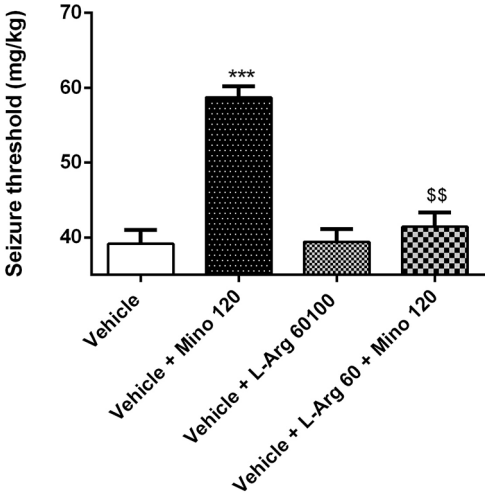


Fig. 5. Effect of pretreatment with L-arginine on the anticonvulsant effect of minocycline: effect of L-arginine (L-arg) administration (60 mg/kg) with saline or minocycline (Mino) (120 mg/kg) on the clonic seizure threshold in pentylenetetrazole (PTZ) injected mice. Values are expressed as the mean ± SEM analyzed by one-way ANOVA followed by Tukey's multiple comparison test (n = 6 for all groups). ***P < 0.001 versus vehicle (saline) injected group and $^{\$\$}P$ < 0.01 versus the minocycline-treated group. PTZ was administrated at a constant rate of 1 mL/min.



decrease in hippocampal nitrite levels in comparison with the animals receiving saline/Tween80 (P < 0.05) (Fig. 7B). Also, co-administration of MK-801 with minocycline caused a significant decrease in hippocampal nitrite levels in comparison with the saline/Tween80-treated group (P < 0.05) (Fig. 7B).

Furthermore, we determined the effects of L-arg (60 mg/kg) as well as co-administration of an effective dose of minocycline (120 mg/kg) with L-arg (60 mg/kg) on the hippocampal nitrite level ($F_{[3,12]}$ = 72.17, P < 0.001) (Fig. 8). Results showed that minocycline (120 mg/kg) significantly decreased the nitrite level in the hippocampus in comparison with the vehicle-received group (P < 0.01). Moreover, co-administration of L-arg (60 mg/kg) significantly reversed the effect of the effective dose of minocycline (P < 0.01).

Discussion

The results of the present study showed that acute administration of minocycline exerted an anticonvulsant effect in the PTZ model of seizure. In addition, administration of subeffective doses of the NOS inhibitor L-NAME, the consecutive NOS inhibitor 7-NI, and NMDA antagonists potentiated the anticonvulsant effect of a subeffective dose of minocycline. Furthermore, we found that the iNOS inhibitor aminoguanidine, did not affect the effect of minocycline. The L-arg NO precursor diminished the beneficial effect of minocycline. Moreover, our results demonstrated that co-administration of subeffective doses of NMDA receptor antagonists and NOS inhibitors (L-NAME and 7-NI) with a subeffective dose of minocycline significantly decreased the hippocampal nitrite level.

The augmented seizure threshold subsequent to minocycline treatment is consistent with previous studies describing anticonvulsant properties for minocycline (Wang et al. 2012; Ahmadirad et al. 2014). There is little information on the role of NO and NMDA receptors in the protective effects of minocycline in seizures, so in the present study, we aimed to investigate this interesting relationship.

Wang and colleagues showed the dose-dependent anticonvulsant activity for minocycline against partial seizures in a minimal clonic seizure model (Wang et al. 2012), but they did not determine the underlying mechanisms of minocycline against seizure. Recent experimental studies on seizure have reported that minocycline inhibited neuronal death, suppressed seizure activity, and inhibited neuroinflammation (Heo et al. 2006; Riazi et al. 2008); Galic et al. 2009). Minocycline treatment in a patient with astrocytoma potently reduced the frequency of seizures (Nowak et al. 2012). On the contrary, other evidence showed that minocycline could not exert anticonvulsant effects in a status epilepticus model in rat (Russmann et al. 2016).

Although several molecular targets have been suggested, the detailed mechanisms of anticonvulsant effects of minocycline remain unclear. It has been determined that minocycline potently inhibits microglial activation and inflammatory reactions in the central nervous system (Yenari et al. 2006). Given that pro- and anti-inflammatory mediators are produced at the sites of seizure, it is likely that minocycline via its anti-inflammatory effects exerts anticonvulsant properties (Murashima et al. 2008). The short time of peak effect indicates that minocycline might have other immediate effects on suppressing neuronal excitability as well as

Fig. 6. Effect of pretreatment with *N*-methyl-D-aspartate antagonists on the anticonvulsant effect of minocycline: effect of administration of (A) ketamine (Ket) (0.5 mg/kg) and (B) MK-801 (0.05 mg/kg) with saline or minocycline (Mino) (40 mg/kg) on the clonic seizure threshold in pentylenetetrazole (PTZ) injected mice. Values are expressed as the mean ± SEM analyzed by one-way ANOVA followed by Tukey's multiple comparison test (n = 6 for all groups). $^{**}P < 0.001$ versus the control saline (vehicle) injected group. PTZ was administrated at a constant rate of 1 mL/min.

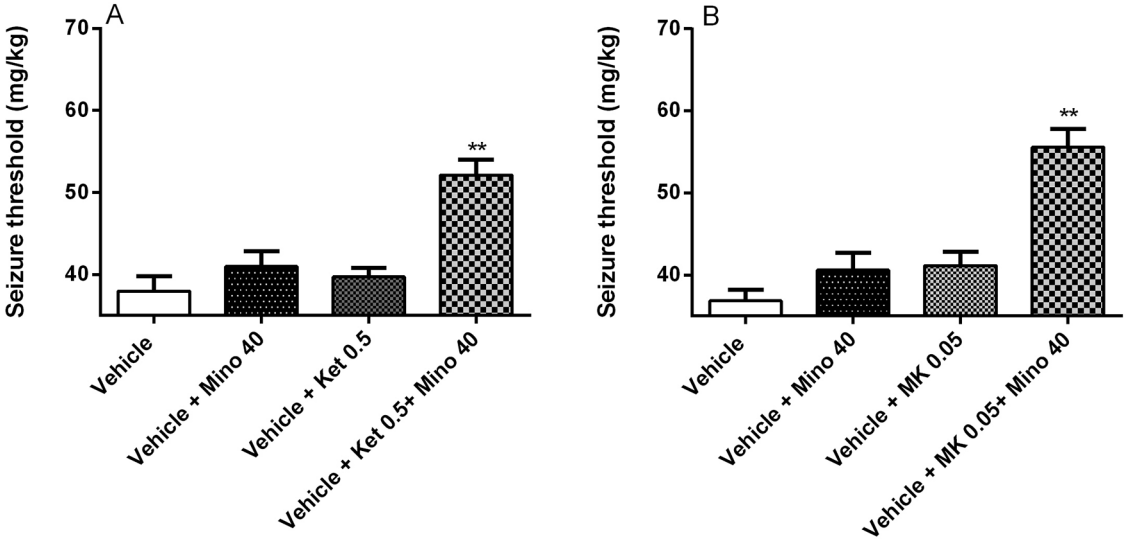
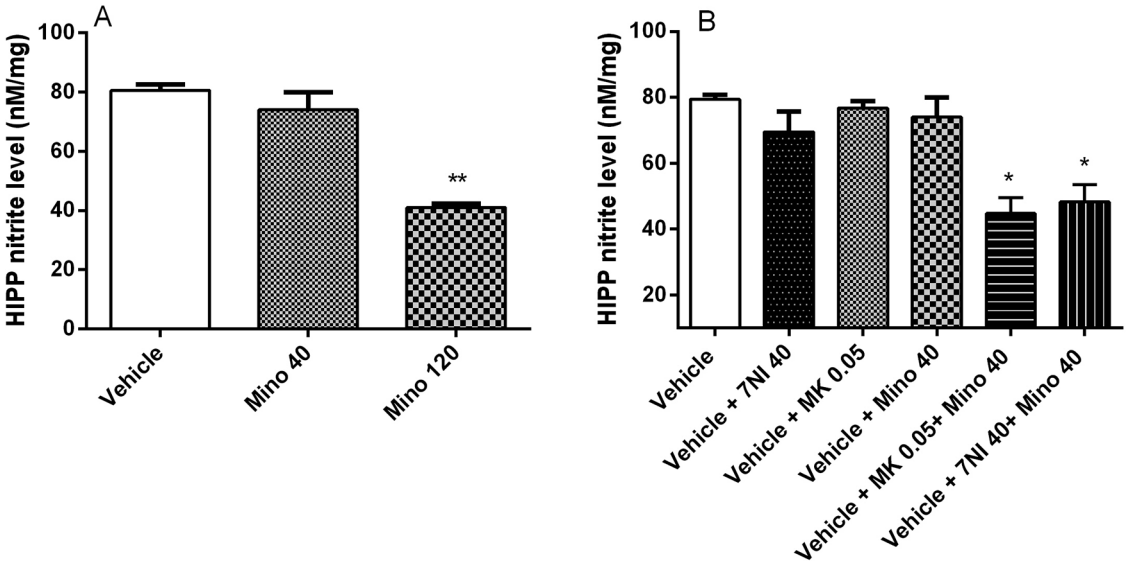


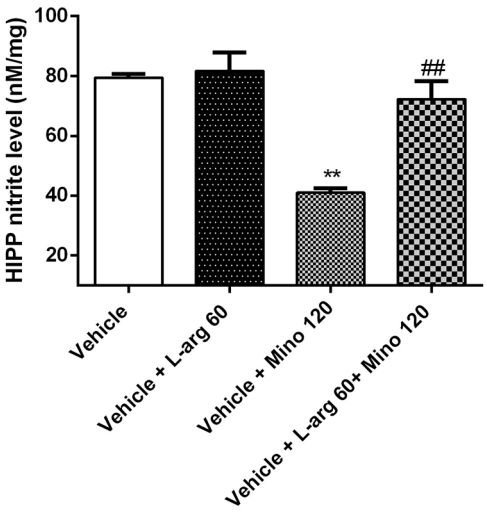
Fig. 7. Effect of minocycline, neuronal nitric oxide synthase inhibitor, and *N*-methyl-D-aspartate receptor antagonist (MK-801) treatment on the hippocampal (HIPP) nitrite level: effect of administration of (A) minocycline (Mino) 40 and 120 mg/kg (60 min before measurement) and (B) 7-nitroindazole (7-NI) (40 mg/kg) and MK-801 (0.05 mg/kg) with Tween80/saline or Mino (40 mg/kg) on the hippocampal nitrite level in mice. Values are expressed as the mean ± SEM analyzed by one-way ANOVA followed by Tukey's multiple comparison test (n = 4 for all groups). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control saline or vehicle (saline and Tween80) injected group.



anti-inflammatory activity (Wang et al. 2012). Considering previous studies that revealed an inhibitory effect for minocycline on the NO pathway (Mora et al. 2014; Saeedi Saravi et al. 2016), we assessed the effects of NOS inhibitors and the NO precursor on the anticonvulsant activity of minocycline in the PTZ model of seizure. We found that NOS inhibitors at their subeffective doses were not able to exert an anticonvulsant effect, while co-administration of these agents with a subeffective dose of minocycline significantly increased the seizure threshold. Furthermore, our findings showed that administration of L-arg reversed the anticonvulsant effect of an effective dose of minocycline. According to determined results on co-administration of NOS inhibitors and the NO precursor with minocycline, it can be suggested that NO may mediate the effect of minocycline on the seizure threshold. An interesting finding of the present study is that the iNOS inhibitor aminoguanidine failed to change the anticonvulsant effect of minocycline, suggesting the noticeable role of nNOS in the anticonvulsant effect of minocycline. Although the literature evidenced that minocycline is a blocker of iNOS expression and activity (Sadowski and Steinmeyer 2001; Huang et al. 2009), this effect might not contribute to the antiseizure activity of this drug in the PTZ-induced seizures, as we conducted experiments on normal animals without an inflammatory condition.

Ample studies have proposed an established role for NO in antiseizure activity of antiepileptic agents (Payandemehr et al. 2014; Shafaroodi et al. 2015). The role of NO in regulating the seizure

Fig. 8. Effect of co-administration of an effective dose of minocycline (Mino) (120 mg/kg) with L-arginine (L-arg) (60 mg/kg) on the hippocampal (HIPP) nitrite level. Values are expressed as the mean ± SEM analyzed by one-way ANOVA followed by Tukey's multiple comparison test ($n = 4$ for all groups). $^{**}P < 0.01$ compared with the vehicle (saline) injected group and $^{\#\#}P < 0.01$ compared with the Mino (120 mg/kg) group.



threshold depends on the type of seizure, source of NO, and other related neurotransmitters (Osonoe et al. 1994; Nidhi et al. 1999). Results of the present study are in line with previous research emphasizimg the role of constitutive NO in mediating the activity of many anticonvulsants (Homayoun et al. 2002*a*; Gholipour et al. 2008; Bahremand et al. 2010*a*; Payandemehr et al. 2014). It has been reported that NO reduced the activity of the GABAergic neurons and increased the seizure susceptibility (Robello et al. 1996). It is believed that NO could mediate the pathophysiology of seizures and seems to be an end product of many excitatory pathways that lead to seizures (Bahremand et al. 2010*b*). In addition, there is a suggested role for abnormal nNOS expression in triggering limbic seizures and delayed excitotoxic damage in the hippocampus (Bagetta et al. 2002). Another study confirmed that abnormally increased nNOS activity triggers mechanisms that evoke seizures (Rajasekaran et al. 2003). It can be suggested that inhibition of nNOS may be associated with the protective effect of minocycline against seizures.

Ionotropic glutamate NMDA receptors are considered as essential modulators for nNOS activity/NO release within the central nervous system (Wu et al. 2001; Filpa et al. 2015). The results of our experiment suggested that the anticonvulsant effect of minocycline could be partially due to decrease in glutamate exocytosis. Previous reports showed that minocycline reversed some NMDA-induced effects (Tikka and Koistinaho 2001; Pi et al. 2004; Garcia-Martinez et al. 2010). The neuroprotective effects and also the central nervous system side effects of minocycline are similar to NMDA receptor antagonists (Munzar et al. 2002). On the other hand, minocycline is thought to inhibit NMDA receptor overexpression (Pu et al. 2013). Minocycline can evidently inhibit NO production (Amin et al. 1996), which acts as an intracellular messenger for NMDA glutamatergic neurotransmission (Bujas-Bobanovic et al. 2000). Therefore, inhibition of NO synthesis might explain the potentiation of effects of minocycline by NMDA blockers. Considering the interaction of minocycline with NMDA receptors, in the current study, we evaluated the effect of co-administration of subeffective doses of minocycline with NMDA receptor blockers (ketamine and MK-801) on the PTZ model of seizure. Our findings showed that administration of subeffective doses of NMDA antagonists significantly increased the seizure threshold following treatment of subeffective doses of minocycline. These results provide clear evidence that NMDA receptors, at least partially, mediated the anticonvulsant effect of minocycline. Preclinical studies have demonstrated that the NMDA receptors are involved in modulation of seizure susceptibility and brain excitation in seizure (Gmiro and Serdyuk 2008; Mareš and Mikulecká 2009). Our results are in line with previous studies declaring that blockade of NMDA receptors decreased susceptibility to seizure development (Borris et al. 2000; Zellinger et al. 2014; Schidlitzki et al. 2017).

Our study showed that the anticonvulsant activity of minocycline, at least in part, mediates through modulation of the glutamatergic and nitrergic pathways. Our results warrant further experimental and clinical investigations to confirm the involvement of the NO/NMDA pathway in the anticonvulsant effect of minocycline. Although the Federal Drug Administration lists convulsion as a side effect for minocycline, this uncommon side effect has been observed with specific doses used for treatment of microbial infections. In this study, we used lower doses of minocycline in comparison with the usual dose used for infectious diseases; however, further investigations are warranted in the future to assess this consideration. Since there is a possible sex difference in the response to neuroprotective agents, the effect of minocycline and its mechanism of action on female mice need to be clarified in future studies. Furthermore, more research in the future should be conducted to understand the mechanisms of action and contribution of other pathways in the antiseizure activity of minocycline.

Conclusions

In conclusion, our results showed that acute minocycline administration enhanced the seizure threshold in the PTZ model of seizure in mice. We demonstrated for the first time that the anticonvulsant effect of minocycline might be related to a decline in iNOS activity and also NMDA receptor inhibition. The novelty of the present work is that an anticonvulsant effect of minocycline is partly associated with its inhibitory effect on the NMDA/nNOS pathways and reduction of NO level in the hippocampus.

Conflict of interest statement

The authors declare that there are no conflicts of interest. The authors declare that there are no sources of funding and support.

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