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Opioidergic and nitrergic systems mediate the anticonvulsant effect of mefloquine and chloroquine on seizures induced by pentylenetetrazol and maximal electroshock in mice

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This study was designed to investigate the involvement of opioidergic/nitrergic systems in the anticonvulsant effect of mefloquine, compared with chloroquine, in mice. Seizures were induced by pentylenetetrazol and maximal electroshock. Mice were randomly subjected to receive mefloquine or chloroquine thirty minutes in advance. The role of opioidergic/nitrergic systems was shown by co-administration of pharmacological intervention and nitrite levels measurement in mice hippocampi. Results indicated that mefloquine (40 mg/kg) and chloroquine (5 mg/kg) significantly decreased the occurrence of tonic hindlimb extension. Also, mefloquine 120 mg/kg and chloroquine 5 mg/kg significantly increased seizure latency and decreased mortality rate. Mefloquine decreased seizure frequency too. Besides, mefloquine (20 mg/kg) and chloroquine (5, 10 mg/kg) significantly increased seizure threshold. Interestingly, L-NAME, 7-NI and naltrexone pre-treatment reversed the anticonvulsant effects of both mefloquine (20 mg/kg) and chloroquine (5 mg/kg). Moreover, co-administration of minimal-effective doses of morphine with mefloquine/chloroquine (both 1 mg/kg) potentiated anticonvulsant effects, which was reversed by naltrexone and endorsed the involvement of opioid receptors. Also, nitrite levels in mice hippocampi remarkably increased after treatment with both mefloquine (20 mg/kg) and chloroquine (5 mg/kg). To conclude, mefloquine could protect the central nervous system against seizures in PTZ/MES-induced models through opioidergic/nitrergic pathways, with similarity to chloroquine effects.

Key words: antimalarial drugs, opioidergic, nitric oxide, pentylenetetrazol, maximal electroshock, seizure, mice

INTRODUCTION

Epilepsy is a chronic disease, which affects more than fifty million people worldwide (Behr et al., 2016; Singh and Trevick, 2016; Beghi, 2020). Although several mechanisms, such as enhancement of hyperpolarization, and sodium/calcium channels modulation, have been reported for the antiepileptic drugs (Rogawski and Löscher, 2004; Macleod and Appleton, 2007; Elger, 2016), further studies are required to investigate novel molecular targets to achieve better symptomatic therapies.

Several studies reported that chloroquine was first introduced as a potent antimalarial drug, and it also has been useful in rheumatoid arthritis (Neill et al., 1973) and systemic lupus erythematosus (Wozniacka et al., 2006). Also, it could affect the central nervous system (CNS), for instance, it shows neuroprotective effects in rats with traumatic brain injury (Cui et al., 2015). Interestingly, during the COVID-19 outbreak this old drug

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attracted much attention as a possible treatment for SARS-CoV-2 (Cirino and Ahluwalia, 2020; Colson et al., 2020). On the other hand, over time, chloroquine-resistant malaria emerged all over the world, and malaria treatment failed in Southeast Asia and large parts of east Africa. It is now believed that this drug will become useless against most of the life-threatening species of plasmodium in a fairly short time (Arrow et al., 2004). Therefore, it is necessary to use other types of antimalarial medication, and mefloquine is an appropriate alternative drug, which is now widely used for malaria chemoprophylaxis and treatment in pregnant women and travelers to endemic regions with chloroquine-resistant P. falciparum (Kofi Ekue et al., 1983; Bloechliger et al., 2014; González et al., 2014). Since mefloquine can quickly cross the blood-brain barrier (BBB), reach a high concentration in hippocampi and subcortical areas, and affect the CNS (Lagerie SB De et al., 2004; Sousa et al., 2014; Quinn, 2015), further investigation into its potential neurological effects will be advantageous. In that regard, we decided to evaluate the effects of mefloquine on seizure activity, and the possible involved mechanisms.

Based on several studies, opioidergic and nitric oxide mechanisms are associated with seizure (Lauretti et al., 1994; Kirkby et al., 1996). The lower doses of morphine (an agonist of μ-opioid receptors) enhance the pentylenetetrazol (PTZ)-induced seizure threshold. However, an increase in dosage leads to a decline in the seizure threshold. So, opioidergic system can exert both anti- and pro-convulsant effects (Calabrese, 2008; Shafaroodi et al., 2011). In addition, nitric oxide (NO), as a signaling molecule in the CNS, which is produced from L-arginine by at least three types of nitric oxide synthase (NOS) isoforms; i.e., neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Knowles and Moncada, 1994; Park et al., 2008), is involved in synaptic transmission and plasticity (Bon and Garthwaite, 2003), stroke and neurodegenerative diseases like Alzheimer's and Huntington's diseases (Hoffman, 1991). Also, it is involved in convulsive behavior (Ribeiro et al., 2009), but the exact role of NO in seizure is somewhat controversial and any changes in nitrergic system could consequently affect seizure activities (Kovács et al., 2009). So, nitric oxide might exert both anti- and pro-convulsant effects, based on its concentration in the central nervous system (Kirkby et al., 1996; Calabrese, 2008). Moreover, NOS inhibitors administration might lead to elevating or lowering seizure susceptibility, depending on the kind of enzyme inhibitors, seizure models, and experimental animals (Wojtal et al., 2003). Interestingly, nitric oxide is involved in morphine-induced peripheral analgesia, dependence, and tolerance. Also, co-administration of NOS inhibitors and morphine can inhibit both phases of the modulatory effect of morphine on seizure threshold. So, nitric oxide could be involved in the biphasic effect of morphine in the PTZ model of seizure (Homayoun et al., 2002a; Shafaroodi et al., 2011). Moreover, co-administration of L-NAME (a nonselective inhibitor of nitric oxide synthase) with naltrexone (a nonselective antagonist of opioid receptors) shows additive effects on reversing the seizure threshold. It is also worth mentioning that the elevation of opioid concentration in plasma leads to nitric oxide overproduction, and to increase the seizure threshold. Therefore, opioidergic system and NO signaling have some interactions with each other, and both are involved in seizure threshold alterations (Homayoun et al., 2002b).

Taking all the above, this study aimed to clarify the possible role of opioidergic and nitrergic systems in the anticonvulsant effects of mefloquine in comparison with chloroquine effectiveness, on PTZ-induced seizure threshold, generalized tonic-clonic seizures, and maximal electroshock test, by co-administration of pharmacological intervention and nitrite levels measurement in mice hippocampi.

METHODS

Reagents

Mefloquine hydrochloride (MQ) was purchased from Macleods Pharmaceuticals, India. L-NAME (N-nitro-L-arginine methyl ester hydrochloride, a nonselective inhibitor of nitric oxide synthase (NOS)) was purchased from Alexis Biochemicals, USA. Chloroquine diphosphate (CQ), pentylenetetrazol (PTZ, a GABA receptor antagonist), and 7-nitroindazole (7-NI, selective inhibitor of neuronal NOS) were purchased from Sigma, USA. Naltrexone hydrochloride (a nonselective antagonist of opioid receptors) and morphine sulfate (an agonist of μ-opioid receptors) were purchased from Iran Darou Pharmaceutical Co, Iran. All drugs were dissolved in sodium chloride 0.9%, and stirred to improve the dissolution rate. Pentylenetetrazol (0.5%) was injected intraperitoneally (i.p.) and intravenously (i.v.). Other drugs were administered intraperitoneally, in a constant volume of 10 ml/kg body weight. All solutions were prepared freshly on each experimental day.

Subjects

Six to eight-week-old male NMRI mice, weighing 23-33 g, were obtained from the laboratory animal cen-

ter of Pharmacology Department, Tehran University of Medical Sciences. The experiment was conducted in a random manner. After weighing animals, we assigned a unique number to every mouse. Then, we used a random number generator to randomly assigned each number to a control or experimental group (n=8, n refers to the number of animals in each group). Animals were held under a 12/12-h light/dark cycle, with free access to enough food (laboratory pellet chow) and water, in standard polycarbonate cages, under the controlled laboratory conditions at a temperature of 23 ± 3°C and humidity of 50 ± 10%. To decrease diurnal variations, behavioral experiments were done at the fixed hours each day. In this study, each mouse was tested once only.

The ethical and legal approval was obtained from the ethics committee of the Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1398.363) before the start of the study, and also the ARRIVE Guidelines have been followed in this research. Furthermore, all experiments were performed in accordance with the institutional guidelines for animal care and use (Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran) and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Procedures

In the present study, seizures were induced by pentylenetetrazol (i.v. and i.p.) and maximal electroshock (MES). The onset of generalized tonic-clonic seizures, the number of seizure attacks, mortality rate, the occurrence of tonic hindlimb extension (THLE), clonic seizure threshold, and nitrite levels in mice hippocampi were assessed as outcome measures. The dose selection method was based on the pilot phase of the experiments and previous animal studies. The injection time schedule was set based on the plasma peak levels and half-life of each drug. Due to overt behavioral seizure activity, the investigators could not be blinded to whether animal was injected with PTZ or not. However, only one of them was aware of the treatment group allocation, the outcome assessment, and the data analysis.

Evaluation of seizure threshold induced by intravenous pentylenetetrazol

For this purpose, a 30-gauge butterfly needle was inserted into the lateral tail vein of the mouse, then the unrestrained animal was connected to the infusion pump, and PTZ solution (0.5%) was slowly infused into the vein with a constant rate of 1 ml/min. The infusion was abruptly discontinued when the first forelimb clonus happened and was normally followed by full clonus of the body. For each animal, the seizure threshold; i.e., the minimum dose of PTZ (mg/kg of body weight) that induced general clonus, was calculated from infusion rate, the time of infusion, body weight, and the concentration of PTZ. In experiment 1, animals received acute injection of different doses of mefloquine (1, 2.5, 5, 10, 20, 40, 80, 120, 200 mg/kg, i.p.) and chloroquine (1, 2.5, 5, 10, 20, 80 mg/kg, i.p.) thirty minutes prior to PTZ infusion. Based on these results, the most effective doses of mefloquine and chloroquine were selected for subsequent experiments. In experiment 2, to determine whether the anticonvulsant effects of mefloquine and chloroquine were mediated through the nitrergic system, animals received i.p. injection of L-NAME (a non-selective inhibitor of NOS, 10 mg/kg) or 7-NI (nNOS selective inhibitor, 30 mg/kg), alone or thirty minutes before the effective anticonvulsant dose of mefloquine (20 mg/kg) or chloroquine (5 mg/kg). In experiment 3, to study the role of the opioidergic system in the anticonvulsant effects of mefloquine and chloroquine, naltrexone (a non-selective antagonist of opioid receptors, 1 mg/kg, i.p.) was acutely administered alone or fifteen minutes before the effective anticonvulsant dose of mefloquine (20 mg/kg) or chloroquine (5 mg/kg). Besides, to provided further clarity on the role of the opioidergic system, lower doses of morphine (an agonist of μ-opioid receptors, 0.5, 1, 2 mg/kg, i.p.) were injected to mice. Then, minimal-effective doses of morphine (0.5 mg/kg, i.p.) and mefloquine/chloroquine (both 1 mg/kg, i.p.) were co-administrated. In another group, animals also received naltrexone (1 mg/kg, i.p.) fifteen minutes before the mentioned drugs.

Evaluation of generalized tonic-clonic seizures induced by intraperitoneal PTZ

In this model of seizure, animals received different doses of mefloquine (1, 2.5, 5, 10, 20, 40, 80, 120, 200 mg/kg, i.p.) and chloroquine (1, 2.5, 5, 10, 20, 80 mg/kg, i.p.) thirty minutes prior to PTZ (80 mg/kg, i.p.). Seizure variables, including latency of seizures (the time lag between PTZ administration to the onset of first anterior limbs myoclonus), frequency of seizures (the number of clonic seizure attacks), and mortality rate were observed during thirty minutes.

Treatment groups received different doses of mefloquine (5, 20, 40 mg/kg, i.p.) and chloroquine (2.5,

5 mg/kg, i.p.) thirty minutes before the test. To improve electrical contact, the animal's ears were moistened by saline before the attachment of ear clip electrodes. The occurrence of THLE and mortality rate following maximal electroshock (frequency 50 Hz, current 50 mA, shock duration 1 s) were assessed for each mouse.

Measurement of nitrite levels in mice hippocampi

To measure the nitrite concentration using the Griess reaction, mice hippocampi in control group and mefloquine/chloroquine-treated groups were dissected, and stored at -80°C. Then, samples were homogenized to perform the test, and after centrifugation at 4000 rpm (Universal 320R centrifuge, Hettich), the supernatants were kept to assay nitrite levels. Each well was loaded with 100 µl of sample and mixed with the same volume of Griess reagent. After fifteen minutes of incubating at room temperature, the absorbance was recorded at 540 nm. Nitrite levels were determined based on a standard sodium nitrite curve and normalized to the weight (mg) of protein in the homogenized sample.

Statistical analysis

To determine the statistical significance of differences between the data of seizure threshold, latency and frequency of seizures, and hippocampal nitrite levels, one-way ANOVA followed by Tukey's multiple comparison test was performed *via* GraphPad Prism 8 software, and results were presented as mean ± standard error of the mean (S.E.M). To analyze the occurrence of tonic hindlimb extension and mortality rate, Chi-square test followed by Fisher's exact analysis (IBM SPSS Statistics 22 software) was used, and results were shown by percentage. In this study P-values less than 0.05 were considered as indicative of significance.

RESULTS

Effects of different doses of mefloquine and chloroquine on seizure threshold

As it is shown in Fig. 1A administration of different doses of mefloquine (1, 2.5, 5, 10, 20, 40, 80, 120, 200 mg/kg, i.p.) thirty minutes prior to PTZ increased the seizure threshold at doses of 1, 2.5 mg/kg (P<0.05); 5, 10 mg/kg (P<0.01); 20 mg/kg (P<0.001); 40 mg/kg (P<0.01) and 80 mg/kg (P<0.05), compared to control group. Fig. 1B illustrates that different doses of chloroquine (1, 2.5, 5, 10, 20, 80 mg/kg, i.p.) administered thirty minutes before PTZ, affected the seizure threshold. Chloroquine at doses of 1 mg/kg (P<0.05); 2.5 mg/kg (P<0.01); 5 and 10 mg/kg (P<0.001) significantly increased seizure threshold compared to control group. The maximum seizure threshold was reached at mefloquine 20 mg/kg (P<0.001), and chloroquine 5 and 10 mg/kg (P<0.001). However, the seizure threshold was decreased in the higher doses of mefloquine (120, 200 mg/kg) and chloroquine (20, 80 mg/kg).

Effects of nitric oxide synthase inhibitors on the anticonvulsant effects of mefloquine and chloroquine

Fig. 2A shows that pre-treatment with L-NAME (10 mg/kg; i.p.) and 7-NI (30 mg/kg; i.p.) thirty minutes before mefloquine (20 mg/kg; i.p.) significantly reversed the seizure threshold compared to mefloquine 20 mg/kg-treated group (P<0.01 and P<0.05, respectively). According to Fig. 2B pre-treatment with L-NAME and 7-NI thirty minutes before chloroquine (5 mg/kg; i.p.) significantly decreased the seizure threshold compared to chloroquine 5 mg/kg-treated group (P<0.001). The administration of L-NAME 10 mg/kg and 7-NI 30 mg/kg alone, had no significant effect on seizure threshold.

Effects of agonist/antagonist of opioid receptors on the anticonvulsant effects of mefloquine

As it is shown in Fig. 3A administration of naltrexone (1 mg/kg; i.p.) fifteen minutes before the effective dose of mefloquine (20 mg/kg; i.p.) significantly reversed the seizure threshold compared to mefloquine 20 mg/kg-treated group (P<0.01). Fig. 3B shows that the administration of lower doses of morphine (0.5, 1, 2 mg/kg; i.p.) significantly increased seizure threshold compared to control group (P<0.05, P<0.001 and P<0.05, respectively). Fig. 3C illustrates that the co-administration of minimal-effective dose of mefloquine (1 mg/kg; i.p.) and morphine (0.5 mg/kg; i.p.) potentiated the anticonvulsant effects and significantly increased seizure threshold compared to control group (P<0.001), which was reversed by naltrexone (1 mg/kg; i.p.) pre-treatment compared to mefloquine 1 plus morphine 0.5 mg/kg-treated group (P<0.001). The administration of naltrexone itself had no significant effect on seizure threshold.

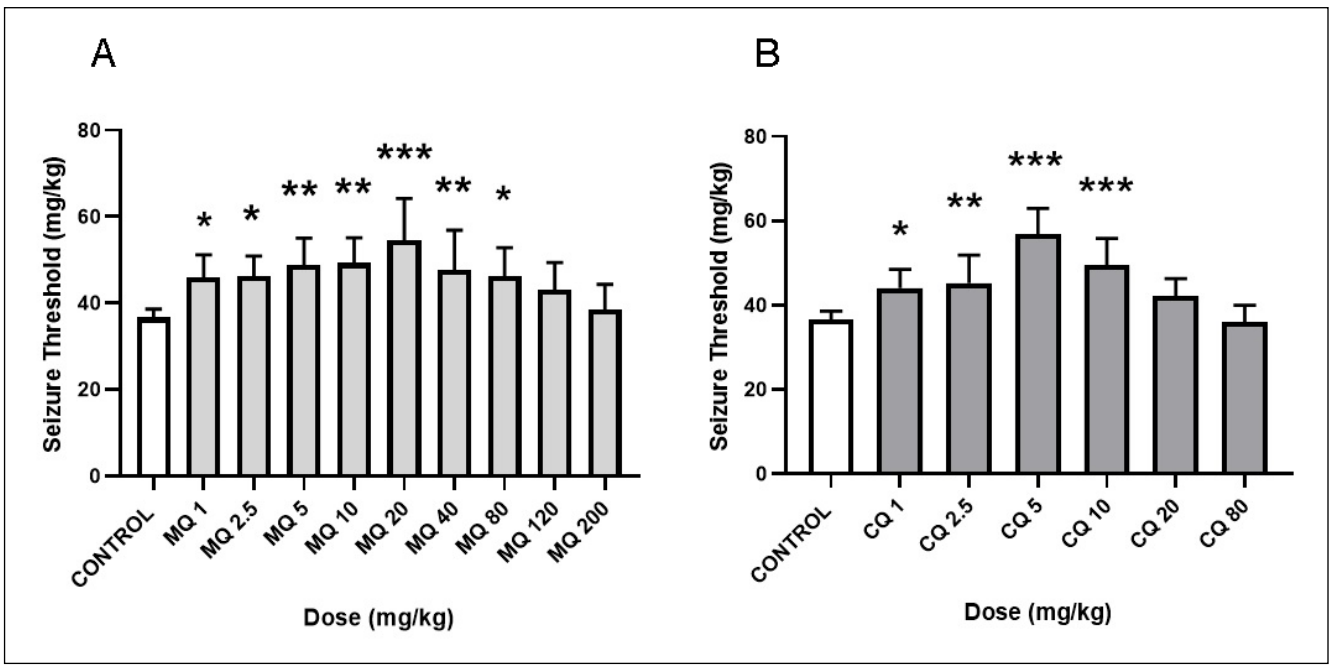

Fig. 1. The effects of different doses of (A) mefloquine (MQ) and (B) chloroquine (CQ) on seizure threshold induced by intravenous pentylenetetrazol in mice. Mefloquine and chloroquine were administered intraperitoneally, thirty minutes prior to PTZ infusion. Data were expressed as mean ± S.E.M. of seizure threshold. * P<0.05; ** P<0.01 and *** P<0.001, compared to the control group, according to one-way ANOVA, followed by Tukey's multiple comparison test.

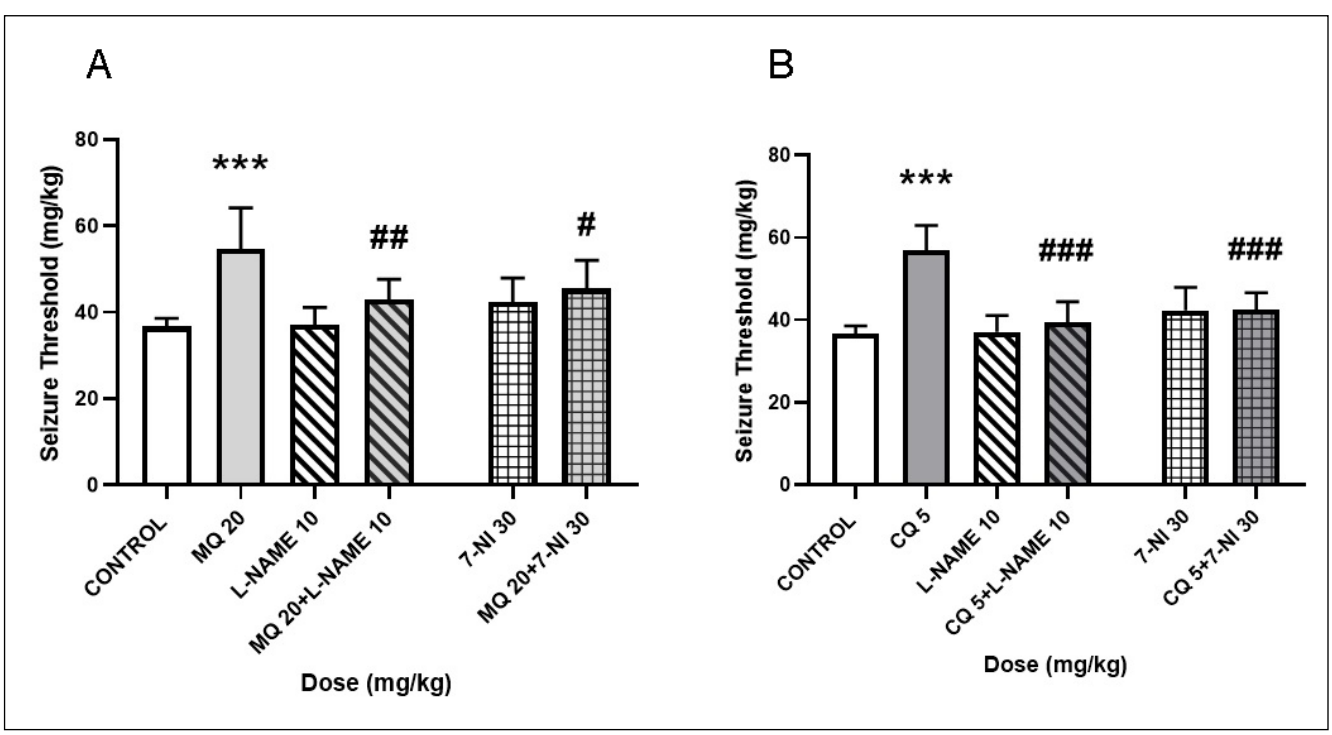

Fig. 2. The effects of nitric oxide synthase inhibitors pre-treatment on seizure threshold elicited by intravenous pentylenetetrazol in mice treated with the effective anticonvulsant dose of mefloquine (MQ) / chloroquine (CQ). L-NAME (10 mg/kg, i.p.) and 7-NI (30 mg/kg, i.p.) were injected thirty minutes before (A) MQ (20 mg/kg, i.p.) and (B) CQ (5 mg/kg. i.p). Data were expressed as mean ± S.E.M. of seizure threshold. *** P<0.001, compared to control group, # P<0.05 and ## P<0.01, compared to the mefloquine (20 mg/kg)-treated group, ### P<0.001, compared to the chloroquine (5 mg/kg)-treated group, according to one-way ANOVA, followed by Tukey's multiple comparison test.

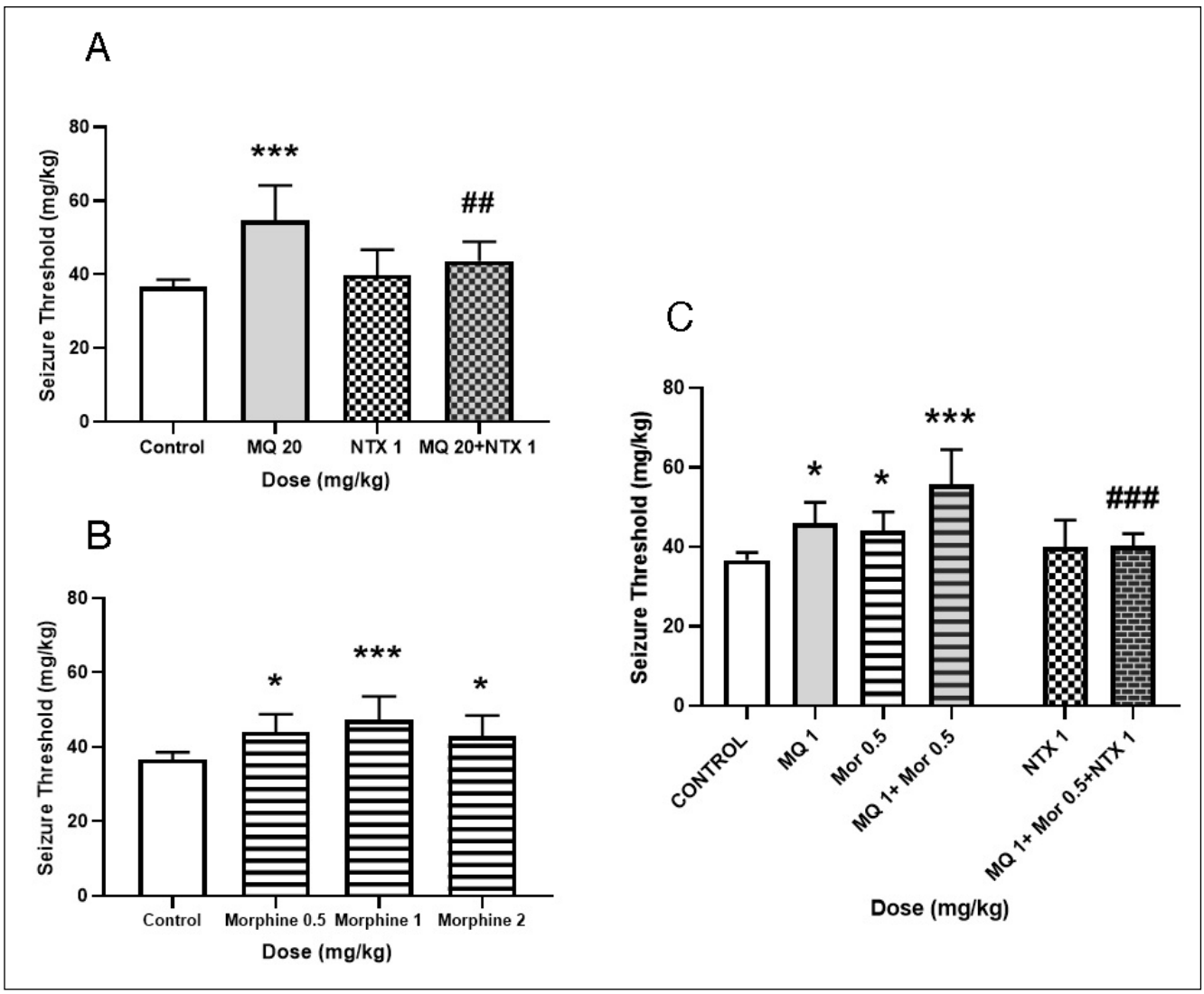

Fig. 3. The effect of the opioidergic system on seizure threshold induced by intravenous pentylenetetrazol in mice treated with mefloquine (MQ). (A) Naltrexone (NTX; 1 mg/kg; i.p.) was administered fifteen minutes before the effective anticonvulsant dose of MQ (20 mg/kg). (B) The lower doses of morphine were administered sixty minutes before PTZ. (C) Co-administration of subeffective doses of MQ (1 mg/kg; i.p.) with morphine (Mor; 0.5 mg/kg; i.p). In addition, naltrexone (1 mg/kg; i.p.) was injected before the mentioned drugs. Data were expressed as mean ± S.E.M. of seizure threshold. * P<0.05 and *** P<0.001, compared to control group, ## P<0.01, compared to the mefloquine (20 mg/kg)-treated group and ### P<0.001, compared to MQ (1 mg/kg) plus morphine (0.5 mg/kg)-treated group, according to one-way ANOVA, followed by Tukey's multiple comparison test.

Effects of agonist/antagonist of opioid receptors on the anticonvulsant effects of chloroquine

According to Fig. 4A administration of naltrexone (1 mg/kg; i.p.) fifteen minutes before the effective anticonvulsant dose of chloroquine (5 mg/kg; i.p.) significantly reversed seizure threshold compared to chloroquine 5 mg/kg-treated group (P<0.001). Fig. 4B shows that the administration of lower doses of morphine (0.5, 1, 2 mg/kg; i.p.) significantly increased seizure threshold compared to control group (P<0.05, P<0.001 and P<0.05, respectively). As it is shown in Fig. 4C co-administration of minimal-effective dose of chloroquine (1 mg/kg; i.p.) with morphine (0.5 mg/kg; i.p.) potentiated the anticonvulsant effects and significantly increased seizure threshold compared to control group (P<0.01), but naltrexone (1 mg/kg; i.p.) pre-treatment significantly reversed the seizure threshold compared to chloroquine 1 plus morphine 0.5 mg/kg-treated group (P<0.001). Naltrexone itself did not have significant effects on seizure threshold.

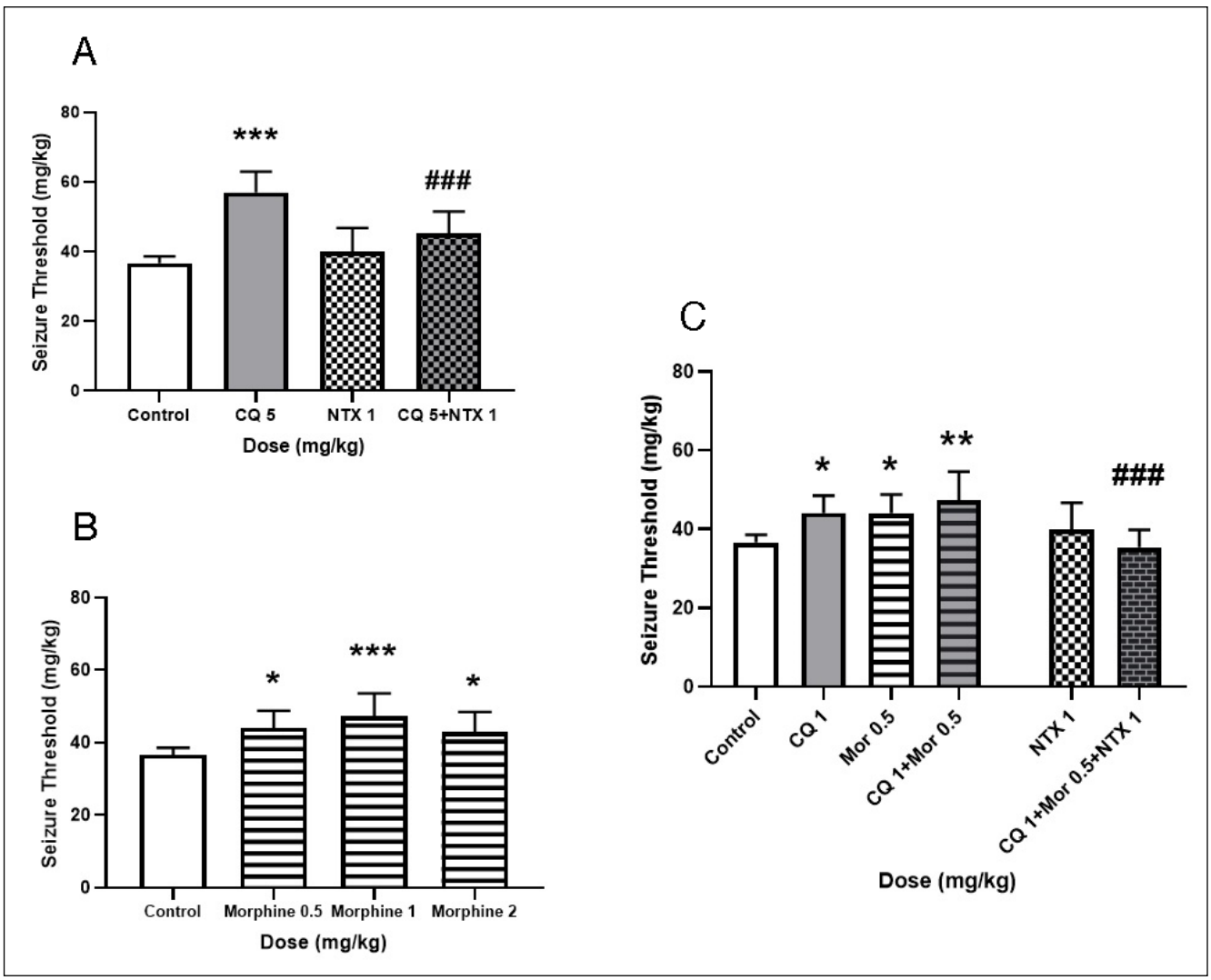

Fig. 4. The effect of the opioidergic system on seizure threshold elicited by intravenous pentylenetetrazol in mice treated with chloroquine (CQ). (A) Naltrexone (NTX; 1 mg/kg; i.p.) was administered fifteen minutes before the effective anticonvulsant dose of CQ (5 mg/kg). (B) The lower doses of morphine were administered sixty minutes before PTZ. (C) Co-administration of subeffective doses of CQ (1 mg/kg; i.p.) and morphine (Mor; 0.5 mg/kg; i.p.). In addition, naltrexone (1 mg/kg, i.p.) was injected before the mentioned drugs. Data were expressed as mean ± S.E.M. of seizure threshold. * P<0.05; ** P<0.01 and *** P<0.001, compared to control group. ### P<0.001, compared to the chloroquine (5 mg/kg)-treated group, and CQ (1 mg/kg) plus morphine (0.5 mg/kg)-treated group, according to one-way ANOVA, followed by Tukey's multiple comparison test.

Effects of different doses of mefloquine on latency and frequency of seizures in generalized tonic-clonic seizures (GTCS)

As it is shown in Fig. 5A mefloquine administration thirty minutes before PTZ (80 mg/kg, i.p) significantly delayed the onset of GTCS (20, 40 mg/kg (P<0.05); 80 mg/kg (P<0.01); 120 mg/kg (P<0.001); 200 mg/kg (P<0.05)). Also, Fig. 5B indicates that the administration of mefloquine before PTZ (80 mg/kg, i.p.) significantly decreased the number of seizure attacks at doses of 80 mg/kg (P<0.01), 120 mg/kg (P<0.001) and 200 mg/kg (P<0.01), compared to control group.

Effects of different doses of chloroquine on latency and frequency of seizures in generalized tonic-clonic seizures

As it is shown in Fig. 6A various doses of chloroquine (1, 2.5, 5, 10, 20, 80 mg/kg, i.p.) were administrated thirty minutes before PTZ (80 mg/kg, i.p.). Chloroquine 5 mg/kg significantly prolonged the onset of GTCS (P<0.01), but it did not alter the frequency of seizures (Fig. 6B).

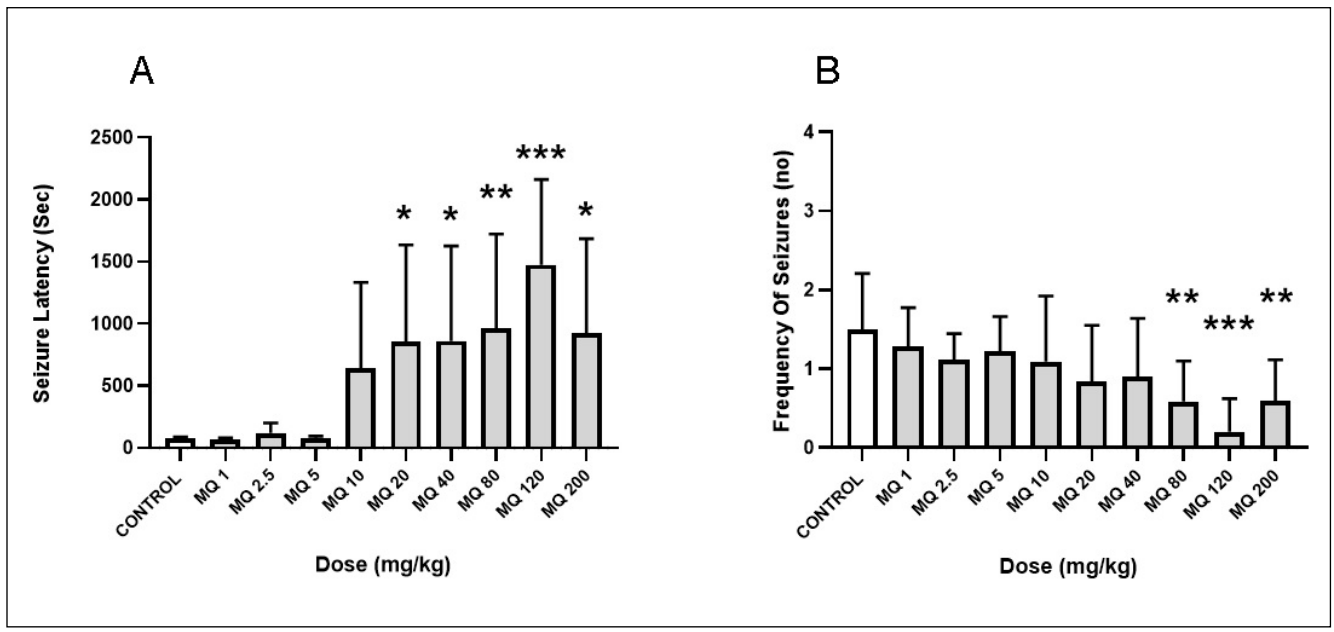

Fig. 5. The effects of different doses of mefloquine (MQ) on (A) seizure latency and (B) the number of seizure attacks in the generalized tonic-clonic seizure. Mefloquine was injected intraperitoneally, thirty minutes prior to PTZ (80 mg/kg; i.p.). Data were expressed as mean ± S.E.M., * $P<0.05$; ** $P<0.01$ and *** $P<0.001$, compared to the control group, according to one-way ANOVA, followed by Tukey's multiple comparison test.

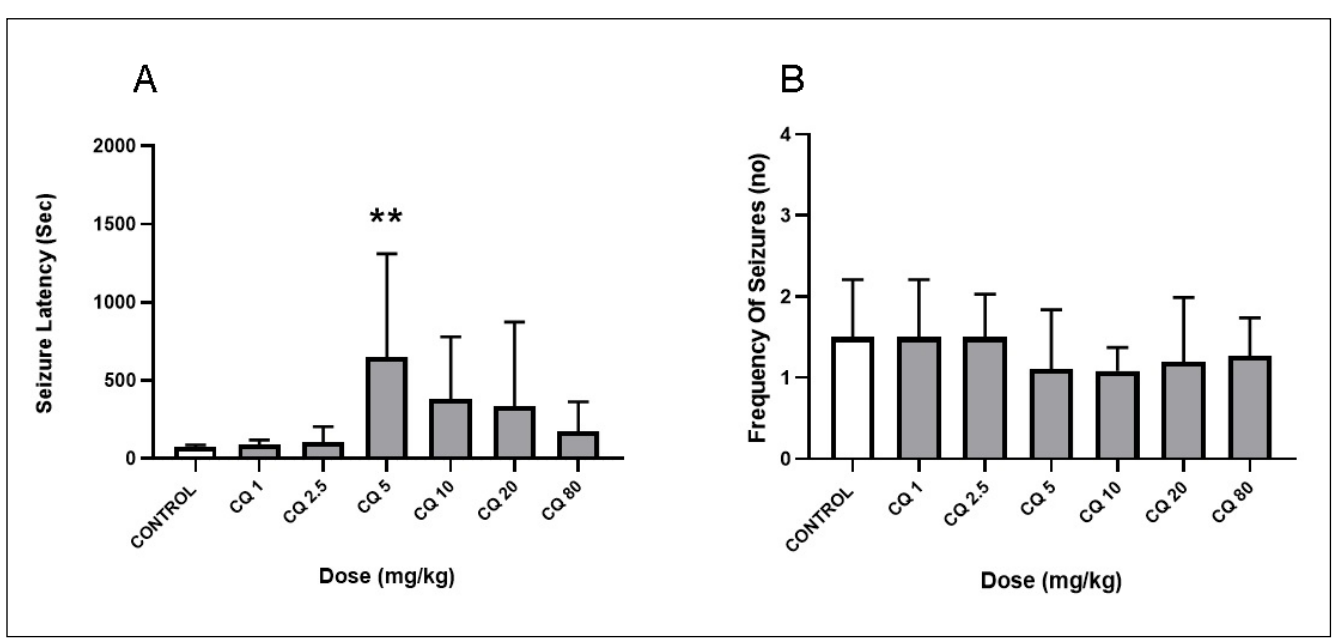

Fig. 6. The effect of various doses of chloroquine (CQ) on (A) seizure latency and (B) the number of seizure attacks in the generalized tonic-clonic seizure. Chloroquine was administrated intraperitoneally, thirty minutes before PTZ (80 mg/kg; i.p.). Data were expressed as mean ± S.E.M., ** $P<0.01$; compared to the control group, according to one-way ANOVA, followed by Tukey's multiple comparison test.

Effects of different doses of mefloquine and chloroquine on mortality rate in generalized tonic-clonic seizures

According to the Table 1 mortality rate is affected by different doses of mefloquine and chloroquine. Mefloquine 1, 2.5, 5, 20, 80 mg/kg (P<0.05); 120 mg/kg (P<0.01), and chloroquine 5, 10 mg/kg (P<0.01 and P<0.05, respectively) significantly increased the survival rate compared to control group. Chloroquine (5 mg/kg) and mefloquine (120 mg/kg) had the most preventive effect on mortality rate.

Table 1. Effects of mefloquine and chloroquine on survival rate in PTZ-induced generalized tonic-clonic seizure in mice.

Groups (*versus* Control)	Survival (*versus* 30%)	P-value
chloroquine 1 mg/kg	60%	0.185 (ns)
chloroquine 2.5 mg/kg	66.7%	0.128 (ns)
chloroquine 5 mg/kg	90%	0.010 (**)
chloroquine 10 mg/kg	83.3%	0.017 (*)
chloroquine 20 mg/kg	70%	0.089 (ns)
chloroquine 80 mg/kg	45.5%	0.392 (ns)
mefloquine 1 mg/kg	88.9%	0.015 (*)
mefloquine 2.5 mg/kg	88.9%	0.015 (*)
mefloquine 5 mg/kg	88.9%	0.015 (*)
mefloquine 10 mg/kg	58.3%	0.185 (ns)
mefloquine 20 mg/kg	75%	0.046 (*)
mefloquine 40 mg/kg	57.1%	0.185 (ns)
mefloquine 80 mg/kg	84.6%	0.012 (*)
mefloquine 120 mg/kg	90%	0.010 (**)
mefloquine 200 mg/kg	60%	0.185 (ns)

Data represent the percentage of survival after acute administration of various doses of chloroquine and mefloquine in mice with generalized tonic-clonic seizures induced by PTZ (80 mg/kg, i.p.). Chloroquine (5 mg/kg) and mefloquine (120 mg/kg) had the most inhibitory effects on mortality. * P<0.05; ** P<0.01 and not statistically significant (ns) P>0.05, compared to control group, according to Chi-Square Tests, Fisher's Exact Test.

Effects of different doses of mefloquine and chloroquine on the occurrence of tonic hindlimb extension in maximal electroshock model

According to the Table 2 the occurrence of THLE induced by maximal electroshock significantly decreased by chloroquine (5 mg/kg, P<0.01) and mefloquine (5, 20 mg/kg, P<0.01; 40 mg/kg, P<0.001) compared to control group. Mefloquine 40 mg/kg and chloroquine 5 mg/kg had the most protective effects against THLE.

Table 2. Effects of mefloquine and chloroquine on the occurrence of tonic hind limb extension (THLE) induced by maximal electroshock in mice.

Groups (*versus* Control)	THLE Protection (*versus* 7.1%)	P-value
chloroquine 2.5 mg/kg	28.6%	0.163 (ns)
chloroquine 5 mg/kg	64.3%	0.002 (**)
mefloquine 5 mg/kg	57.1%	0.006 (**)
mefloquine 20 mg/kg	57.1%	0.006 (**)
mefloquine 40 mg/kg	78.6%	0.000 (***)

Data represent the percentage of THLE protection by acute treatment with different doses of mefloquine and chloroquine in mice with maximal electroshock-induced seizure. Chloroquine (5 mg/kg) and mefloquine (40 mg/kg) had the most protective effects against THLE. ** P<0.01; *** P<0.001 and not statistically significant (ns) P>0.05, compared to control group, according to Chi-Square Tests, Fisher's Exact Test.

Effects of different doses of mefloquine and chloroquine on mortality rate in maximal electroshock model

As it is shown in Table 3 neither mefloquine nor chloroquine had significant effect on the mortality rate after electroshock convulsion.

Effects of mefloquine and chloroquine on hippocampal nitrite levels

Fig. 7. shows that the anticonvulsant doses of mefloquine (20 mg/kg) and chloroquine (5 mg/kg) significantly increased nitrite levels in mice hippocampus (P<0.05 and P<0.01, respectively).

Table 3. Effects of mefloquine and chloroquine on survival rate in maximal electroshock-induced seizure in mice.

Groups (*versus* Control)	Survival (*versus* 78.6%)	P-value
chloroquine 2.5 mg/kg	92.9%	0.298 (ns)
chloroquine 5 mg/kg	92.9%	0.298 (ns)
mefloquine 5 mg/kg	85.7%	0.5 (ns)
mefloquine 20 mg/kg	100%	0.111 (ns)
mefloquine 40 mg/kg	92.9%	0.298 (ns)

Data express the percentage of survival in mefloquine/chloroquine-treated mice in maximal electroshock test. Mefloquine and chloroquine did not have significant effects on survival percentage. Not statistically significant (ns) $P>0.05$ compared to control group, according to Chi-Square Tests, Fisher's Exact Test.

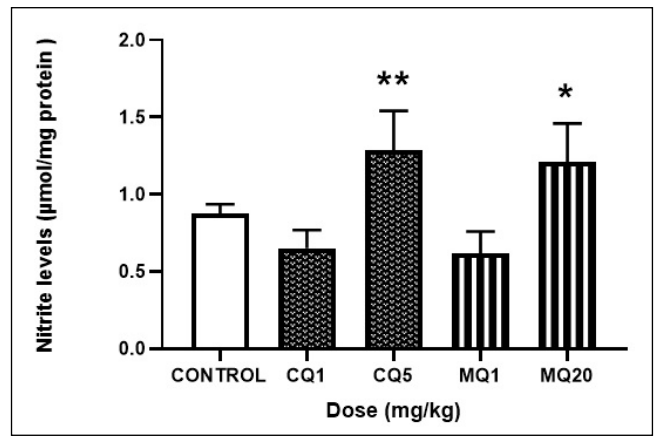

Fig. 7. The effects of different doses of mefloquine (MQ; 1, 20 mg/kg; i.p.) and chloroquine (CQ; 1, 5 mg/kg; i.p.) on nitrite levels in mice hippocampi. Data were expressed as mean ± S.E.M., * $P<0.05$ and ** $P<0.01$; compared to the control group, according to one-way ANOVA, followed by Tukey's multiple comparison test.

DISCUSSION

The present study for the first time investigated that the anticonvulsant effects of mefloquine are mediated by opioidergic/nitrergic systems with similarity to chloroquine effects. Mefloquine significantly increased seizure latency, and decreased the number of seizure attacks and mortality rate in intraperitoneal PTZ-induced seizures. Also, it decreased THLE episodes in MES test. In the same way, chloroquine affected i.p. PTZ- and MES-induced seizures by enhancing seizure latency, and lowering mortality rate and THLE occurrence. Both mefloquine and chloroquine also significantly increased PTZ-induced seizure threshold ($P<0.001$). Furthermore, NOS inhibitors (L-NAME and 7-nitroindazole), and nonselective antagonist of opioid receptors (naltrexone) attenuated the anticonvulsant effects of mefloquine. However, co-administration of subeffective dose of morphine (an agonist of µ-opioid receptors; 0.5 mg/kg) and mefloquine (1 mg/kg) potentiated the anticonvulsant effects. Besides, our data revealed that chloroquine showed the anticonvulsant effects by the same mechanisms. Its anticonvulsant effects were inhibited by L-NAME, 7-NI, and naltrexone pre-treatment, but co-administration of the subeffective dose of morphine potentiated that. Also, the anticonvulsant effects of mefloquine and chloroquine were associated with elevated levels of nitric oxide in mice hippocampus. So, our results demonstrated that mefloquine showed its anticonvulsant effects through opioidergic/nitrergic pathways, in i.p. PTZ-induced generalized tonic-clonic seizure, maximal electroshock test, and PTZ-induced clonic seizure threshold, with similarity to chloroquine.

As previously described (Mandhane et al., 2007), various classical antiepileptic drugs (AEDs) were used as reference drugs in animal models of seizure. The comparison of mefloquine/chloroquine to such known anticonvulsants could provide a more reliable evaluation of the anticonvulsant-like effects of antimalarial drugs. Based on our data (Fig. 1), mefloquine 20 mg/kg and chloroquine 5 mg/kg significantly increased the clonic seizure threshold to nearly 54.60 mg/kg and 55.17 mg/kg, respectively, compared to the control group (37.31 mg/kg). The comparison between our results and the mentioned study, which has investigated that different AEDs such as sodium valproate (200 mg/kg) increased the clonic seizure threshold to 66.83 mg/kg, *versus* 44.17 mg/kg in the control group, could support the efficient anticonvulsant effects of mefloquine and chloroquine. Also, with similarity to other reference anticonvulsants such as diazepam (1 mg/kg) and phenytoin (25 mg/kg) (Rashidian et al., 2017; Mombeini et al., 2020), we showed protective effects against seizures induced by PTZ and maximal electroshock for mefloquine and chloroquine.

Chloroquine had been the drug of choice in malaria treatment, but nowadays it is mostly replaced with mefloquine, because of the global spread of chloroquine-resistant malaria (Bloechliger et al., 2014). Mefloquine can affect the central nervous system, as it can quickly cross the BBB (Lagerie SB De et al., 2004; Sousa et al., 2014; Quinn, 2015), and Naito et al. (2012) reported that it was effective in progressive multi-

focal leukoencephalopathy (PML) treatment. Also, Cui et al. (2015) showed the neuroprotective activity for chloroquine in rats with traumatic brain injury. Previous studies also reported some anticonvulsant properties for chloroquine in chemically induced seizure (N'Gouemo et al., 1994; Hassanipour et al., 2016), which our study is in line with. In addition, to provide further investigation, the present study is the first report on the anticonvulsant effects of chloroquine in i.p. PTZ and MES models of seizure.

Moreover, in this study we investigated the opioidergic/NO pathways as possible mechanisms for anticonvulsant effects of mefloquine as well as chloroquine. Foroutan and co-workers (2015) showed the role of NO in chloroquine-induced scratching in mice, and according to Inan and Cowan (2004) kappa-opioid agonists could suppress scratching induced by chloroquine. Also, Moradi and co-workers (2013) showed that mefloquine could attenuate opioid withdrawal signs in morphine-dependent rats. To provide more supports on the involvement of opioid receptors in the mechanism of antimalarial drugs, Malek et al. (2020) could identify binding sites of interaction between chloroquine and the μ opioid receptor, using molecular docking and molecular dynamic simulation techniques, and they showed that chloroquine, like opioid agonists, could interact to active site cavity of μ opioid receptors.

Several studies indicated the role of the opioidergic system in seizures (Calabrese, 2008; Shafaroodi et al., 2011). Lauretti et al. (1994) reported that opioid drugs exhibited a biphasic dose-response effect on seizure threshold. In addition, nitric oxide is considered to have various effects on seizure activity depending on the type of NOS inhibitors, experimental animals and the model of the seizure (Kirkby et al., 1996; Wojtal et al., 2003). Interestingly, there is a strong interaction between opioidergic and nitrergic systems, which has been shown in various conditions. Haj-Mirzaian et al. (2013) showed that endogenous opioids and NO could induce antidepressant-like effects in cholestatic mice. Homayoun et al. (2002b) also showed the additive effect of opioids and nitric oxide on reversing the seizure threshold in cholestatic mice. Moreover, it was indicated that nitric oxide could mediate both pro- and anti-convulsant effects of morphine (Homayoun et al., 2002a).

Furthermore, we showed that the anticonvulsant effects of mefloquine and chloroquine were associated with an increase in nitrite levels in mice hippocampi, and nNOS could be partly responsible for nitric oxide synthesis. As Ghigo et al. (1998) reported, chloroquine could stimulate NO production in human and murine endothelial cells, and in A172 human glioblastoma cells (Park et al., 2008). Also, Chen et al. (2005) showed the role of iNOS in chloroquine-induced nitric oxide synthesis in C6 glioma cells. Yadav et al. (2018) also showed that mefloquine could normalize nitric oxide levels in serum of streptozotocin/nicotinamide-diabetic rats.

Taken together, our current study indicates that widely-used antimalarial drugs, chloroquine and mefloquine, have also potential antiepileptic properties as they showed positive effects on PTZ- and MES-induced seizures. Therefore, antimalarial drugs might be used as adjunctive therapies in patients with AEDs-resistant epilepsy; however, there are several case reports of retinopathy (Bernstein and Ginsberg, 1964) and severe pruritus (Ajayi, 2019) with antimalarial drugs. Interestingly, hydroxychloroquine, an analogue of chloroquine, which is commonly prescribed in treating rheumatoid arthritis (Rempenault et al., 2020) and recently used to treat SARS-CoV-2 infection (Jorge, 2021), shows a safer toxicity profile and lower risk of ocular toxicity (Lim et al., 2009). Not only is it clinically preferred over chloroquine, but it also has the CNS penetration ability (Ong et al., 2021), so it can feasibly have antiepileptic effects. Undoubtedly, further clinical studies are needed to determine the efficacy and safety of these antimalarial drugs in drug-resistant epileptic patients.

CONCLUSION

This study demonstrated that both mefloquine and chloroquine exerted anticonvulsant effects on seizure threshold, intraperitoneal PTZ-induced seizures, and maximal electroshock test. They significantly increased clonic seizure threshold, and delayed the onset of generalized tonic-clonic seizures. Also, they decreased the number of seizure attacks, mortality rate, and the occurrence of THLE. Moreover, co-administration of mefloquine/chloroquine with pharmacological intervention and measurement of nitrite levels in mice hippocampi revealed for the first time that the anticonvulsant effects of mefloquine could be mediated *via* opioid receptors and nitric oxide pathway, with some similarities to chloroquine.

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