EFFETS OF ETHOSUXIMIDE ON MORPHINE TOLERANCE AND DEPENDENCE IN MICE

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Summary

In the present study, the effects of ethosuximide (an anticonvulsant with T-type calcium channel blocking activity) on the expression and development of morphine antinociceptive tolerance, and naloxone-precipitated abstinence syndrome in morphine dependent mice were investigated. Mice were rendered tolerant and dependent on morphine by repeated administration of morphine. The tail-flick test was used to assess the nociceptive threshold, naloxone-induced jumping to assess the morphine dependence and open field to evaluate the locomotion. Repeated administration of ethosuximide (100, 200 and 400 mg/kg, 2 time/day for 4 day) and also single administration of ethosuximide (100 mg/kg, simultaneously with last dose of morphine) reduced the development and expression of tolerance to the antinociceptive effect of morphine. Repeated administration of ethosuximide (100, 200 and 400 mg/kg, 3 time /day for 3 day) and also single administration of ethosuximide (100, 200 and 400 mg/kg, 30 min before the last dose of morphine) reduced the development and expression of naloxone-induced jumping in dependent mice that was comparable with clonidine (0.1 mg/kg) as positive control. Single (200 and 400 mg/kg) and repeated (only in 400 mg/ kg) administration of ethosuximide reduced the activity of animals in open field test. Ethosuximide in 200 and 400 mg/kg had direct antinociceptive effect that wasn't blocked by naloxone. These results showed that ethosuximide, a relatively selective T-type calcium channel blocker has direct antinociceptive effect, prevents the development and expression of antinociceptive tolerance to morphine and suppress morphine withdrawal syndrome.

Keywords: Ethosuximide; Dependence; Morphine tolerance; Withdrawal; T-type voltage dependent calcium channels; Antinociception

Introduction

Repeated use of opiate drugs, such as morphine, for pain relief leads to the development of tolerance and dependence. Morphine tolerance and dependence are complex phenomena, and several mechanisms including changes in neurotransmitters and their receptors (eg. catecholamines, serotonin, acetylcholine, γ-amino-butyric acid (GABA) and excitatory aminoacids) (1), nitric oxide pathway (2), cAMP (3) and c- fos (4) have been suggested as involved mechanisms. Also a growing body of evidence supports the participation of calcium influx through voltage dependent calcium channels (VDCC) in opioid antinociception, the development of tolerance, and dependence (5). L- and N-type calcium channel blockers enhance the antinociceptive effect of morphine (6, 7) and prevent naloxone-precipitated morphine withdrawal syndrome (8). Also there is a study that showed mibefradil (a T-type VDCC blocker) augments the antinociceptive effects of morphine, prevents the development of antinociceptive tolerance to morphine and suppress morphine abstinence syndrome (5).

On the other hand, several anticonvulsant drugs including gabapentin (9), diazepam (10), felbamate (11), valproate sodium (12) and phenobarbital, (13) also have been shown to be effective in attenuating morphine tolerance and dependence. Meanwhile there is no study about the effect of succinimde derivative ethosuximide, or 2-ethyl-2-methylsuccinimide, on morphine tolerance and dependence. Ethosuximide, an anticonvulsant effective in the treatment of absence epilepsy, has been demonstrated to be a relatively specific T-type channel antagonist (14). Therefore we hypothesized that ethosuximide may affect morphine tolerance and dependence. In the present study, the effects of ethosuximide (as a T-type VDCCs blocker anticonvulsant) on the development of morphine antinociceptive tolerance, and naloxone-precipitated abstinence syndrome in morphine dependent mice were investigated.

Methods

2.1. Animals

Male NMRI mice (25–30 g) were housed in plastic cages in an animal room maintained at 21° ± 2° C on a 12-h dark cycle. Animals had free access to water and food. The experimental protocol was approved by the "Animal Studies Ethics Committee" of the School of Pharmacy, Mashhad University of Medical Sciences and all procedures were carried out in accordance with institutional guidelines for laboratory animal care and use. Each mouse was used only once and each treatment group consisted of 7 animals.

2.2. Drugs

The following drugs were used: ethosuximide (Sigma, Germany), morphine sulphate (Daru Pakhsh, Iran), clonidine (Daru pakhsh, Iran), naloxone hydrochloride (Tolidaru, Iran). The drugs were dissolved in saline and were injected intraperitoneally (i.p.) in a volume of 10 ml/kg, except morphine which was administered subcutaneously (s.c.). The control groups received saline. The doses of drugs used had been shown to be active in previous studies (15, 16, 17).

2.3. Antinociceptive assay

Antinociception was assessed using the radiant heat tail-flick test (Sparko, Co, Iran). Thirty min after drug injections, the tail-flick latencies were measured. A cut off time of 10 s was used to prevent tissue damage. In a part of experiments, Antinociceptive effect of ethosuximide (100, 200 or 400 mg/kg, i.p.), or morphine (7 mg/kg, s.c.) were determined in

different groups of mice using heat tail-flick test. Antinociceptive effect of ethosuximide (400 mg/kg, i.p.), was also evaluated in the presence of naloxone (5 mg/kg, i.p.). In order to evaluate the antinociceptive effect of remained ethosuximide after 4 days administration, ethosuximide (400 mg/kg, i.p. 2time/day) was injected for 4 days and on the next day, antinociceptive activity was assessed.

2.4. Induction and evaluation of morphine tolerance

A 4-day dosing regimen was used for the induction of morphine tolerance (16). Morphine was injected to each mouse at dose 20 mg/kg (s.c.) two times daily (9:00 and 16:30) for 4 days (days 2 to 5). On day 1 and 6, mice were tested for antinociception (tail-flick) 30 min following morphine administration (7 mg/kg; s.c.) and the result compared to determine the intensity of tolerance to antinociceptive effect of morphine.

In one group of animals, in order to test the effect of ethosuximide on expression of morphine tolerance, on day 6, ethosuximide (100 mg/kg, i.p) was injected simultaneously with morphine and mice were tested for antinociception (tail-flick) 30 min following morphine administration (7 mg/kg; s.c.).

In the other group of animals, to evaluate the effect of ethosuximide on development of morphine tolerance, on days2 to 5, ethosuximide (100, 200 or 400 mg/kg, i.p.) was injected simultaneously with morphine (8 doses) and mice were tested for antinociceptive activity 30 min following morphine administration (7 mg/kg; s.c.) on the 6th day.

2.5. Induction and evaluation of morphine dependence

The mice were rendered dependent on morphine, based on the method previously used by Zarrindast (17). Morphine was injected (s.c.) three times daily at 8, 12 and 16 h (50, 50 and 75 mg/kg, respectively) for 3 days. The higher dose of the third daily injection was aimed to minimize any overnight withdrawal. A dose of 50 mg/kg of morphine was also injected on the 4th day. Two hours after the last dose of morphine (50 mg/kg), abstinence was precipitated by injection of naloxone (5 mg/kg, i.p.) and the animals were then placed individually in a Perspex observation cylinder (15 cm diameter, 50 cm height). The number of jumps was recorded immediately after the injection of naloxone over a 30-min period.

One group of animals received either saline (10 ml/kg) or different doses ethosuximide (100, 200 or 400 mg/kg, i.p.), 15 min before naloxone injection, to test the effect of ethosuximide on the expression of withdrawal signs. Clonidine (0.1 mg/kg, i.p.) was used as positive control.

Other group of animals received different doses of ethosuximide (100, 200 or 400 mg/kg, i.p.), or saline, or clonidine (as positive control) simultaneously with morphine for 3 days (9 doses) to test the effect of ethosuximide on the development of dependence on morphine.

2.6. Measurement of locomotor activity

To evaluate the effect of drugs on locomotor activity, all of the animal in dependence test, were subjected to open field test before injection of naloxone. The open-field (100 cm width × 100 cm length × 40 cm height) was divided into a 5 × 5 grid of equally sized squares using black tape. Each mouse was gently placed in the center of the box and activity was scored as a line crossing when a mouse removed all four paws from one square and entered another. Line crossings in the squares were counted for 10 min (18).

2.7. Statistical analysis

Analysis of variance (ANOVAs) followed by Tukey-kramer test were used for analysis of three or more groups of data. Unpaired Student's t-test was used to analysis two groups of data. Differences between means were considered statistically significant if $P < 0.05$. Data were expressed as the mean + SEM**.**

Results

3.1. Antinociceptive effect of ethosuximide

In single dose administration, ethosuximide produced a significant antinociceptive effect in doses 200 and 400 mg/kg but not in dose 100 mg/kg (Fig. 1). The effect of ethosuximide (400 mg/ kg) was comparable with morphine (7 mg/kg). Co-administration of naloxone with ethosuximide didn't have significant influence on antinociceptive effect of ethosuximide. (Fig.3). In repeated administration of ethosuximide (400 mg/kg two dose/day for 4 days), tail-flick test in the day after last administration of ethosuximide, showed no significant antinociceptive effect (Fig. 2).

3.2. Effects of ethosuximide on expression or development of morphine tolerance

Repeated administration of morphine (20 mg/kg 2time/day for 4 days) significantly reduced the antinociceptive effect of morphine in tail-flick test (fig. 4). The effect of repeated doses of ethosuximide (100, 200 or 400 mg/kg 2 time/day for 4 day), on the development of morphine tolerance has been shown in fig 6. Ethosuximide in all doses attenuated the development of tolerance to morphine-induced antinociception (statistically significant in doses 200 and 400 mg/kg). On the other hand, in single dose administration, ethosuximide (100 mg/kg) didn't have any significant effect on the expression of morphine tolerance (fig. 5).

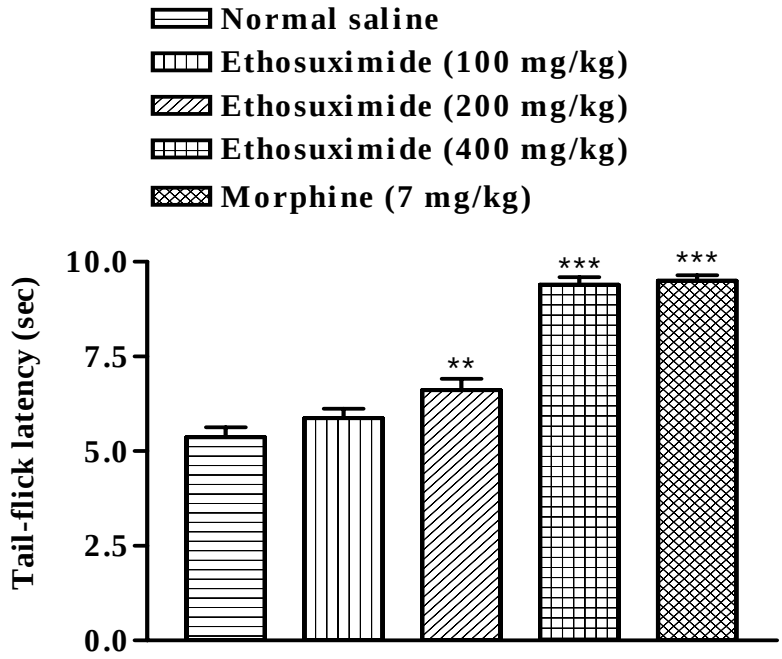


Figure 1. Antinociceptive effect of single dose of ethosuximide in mice. The animals received saline or different doses of ethosuximide (100, 200 or 400 mg/kg) intraperitoneally 30 min before tail-flick test. Each group comprised 7 mice. Data are means + S.E.M. **P < 0.01, ***P < 0.001 different from the saline control group (ANOVA followed by Tukey-kramer test).

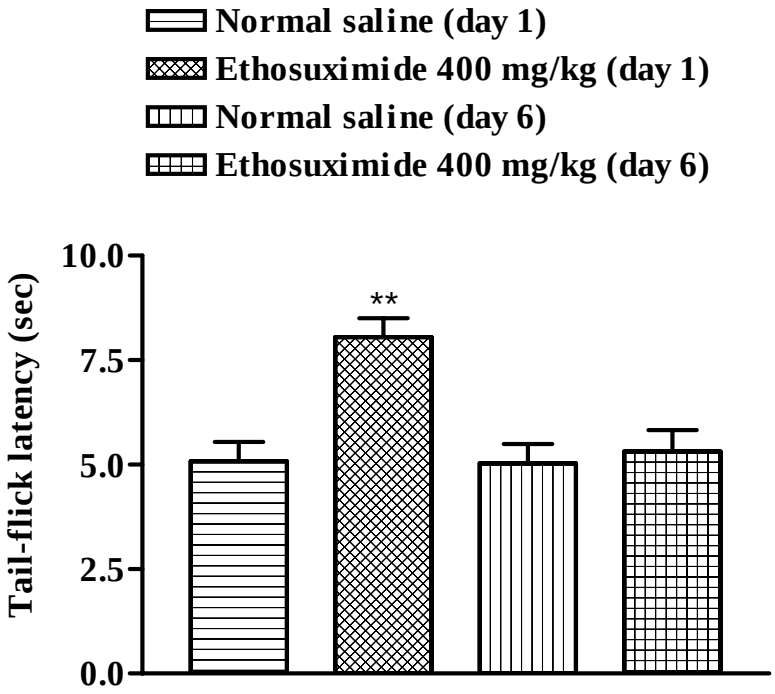


Figure 2. Antinociceptive effect of repeated doses of ethosuximide in mice. The animals received saline or ethosuximide (400 mg/kg) intraperitoneally and the tail-flick test carried out. Then animals received saline or ethosuximide (400 mg/kg, 2 time/day) for 4 days and on next day (day 6) the tail-flick test were carried out to evaluate the remained Antinociceptive effect of ethosuximide one day after last dose. Each group comprised 7 mice. Data are means + S.E.M. **P < 0.01, different from the saline control group (ANOVA followed by Tukey-kramer test).

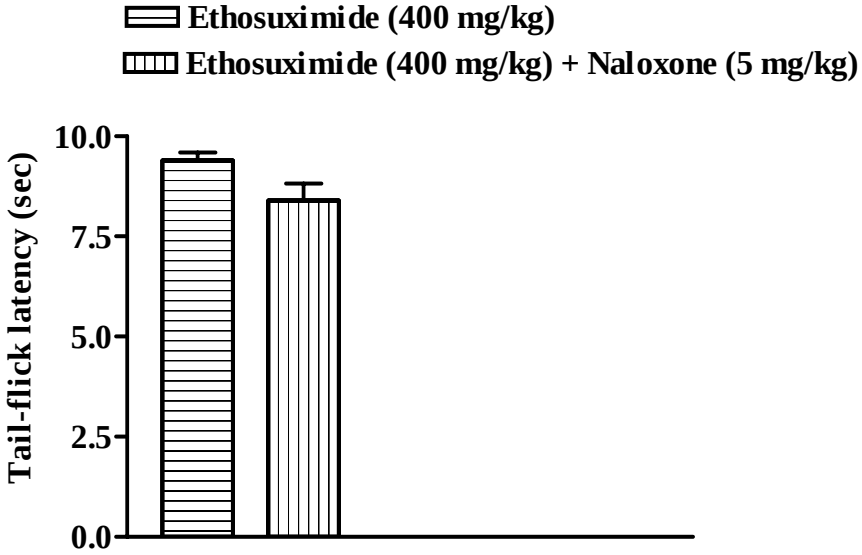


Figure 3. Antinociceptive effect of ethosuximide in the presence of naloxone. The animals received ethosuximide (400 mg/kg i.p.) and naloxone (5 mg/kg, i.p.).30 min before tail-flick test. Each group comprised 7 mice. Data are means + S.E.M. there is not significant difference between groups (unpaired Student's t-test).

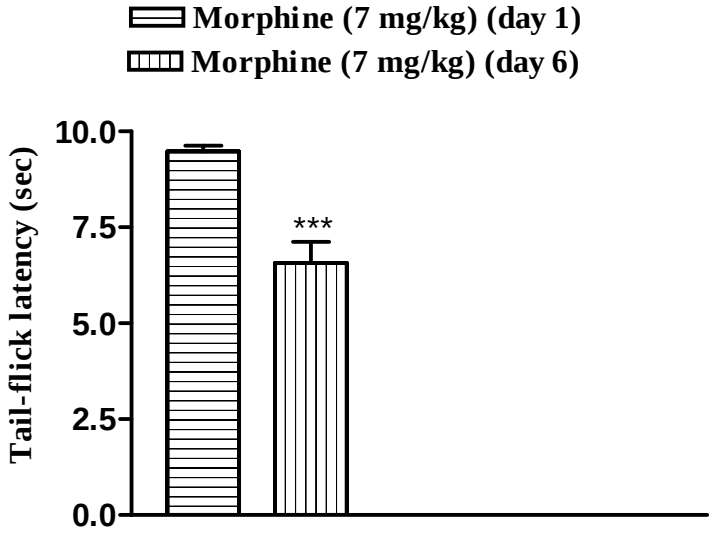


Figure 4. Antinociceptive effect of morphine before and after induction of tolerance. The animals received morphine (7 mg/kg) and tail-flick test carried out. Then animals received morphine (20 mg/kg 2time/day for 4 days) for induction of morphine tolerance. On next day, animals again received morphine (7 mg/kg) and tail-flick test were carried out. Each group comprised 7 mice. Data are means + S.E.M. **P < 0.01, different from the saline control group (unpaired Student's t-test).

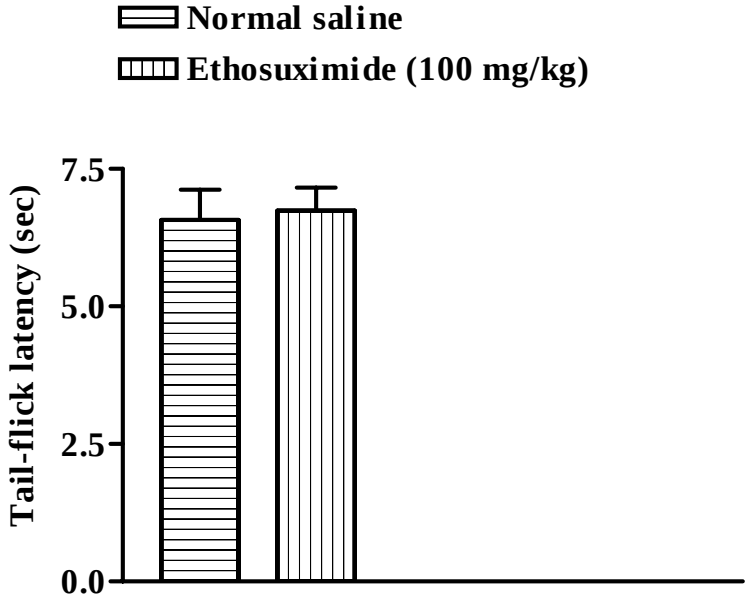


Figure 5. Effect of ethosuximide on expression of morphine tolerance in mice. The animals received morphine (20 mg/kg 2time/day for 4 days) for induction of morphine tolerance. On the next day, animals received ethosuximide (100 mg/kg i.p.) or saline and morphine (7 mg/kg) and tail-flick test were carried out to evaluate the effect of ethosuximide on expression of morphine tolerance. Each group comprised 7 mice. Data are means + S.E.M. there is not significant difference between groups (unpaired Student's t-test).

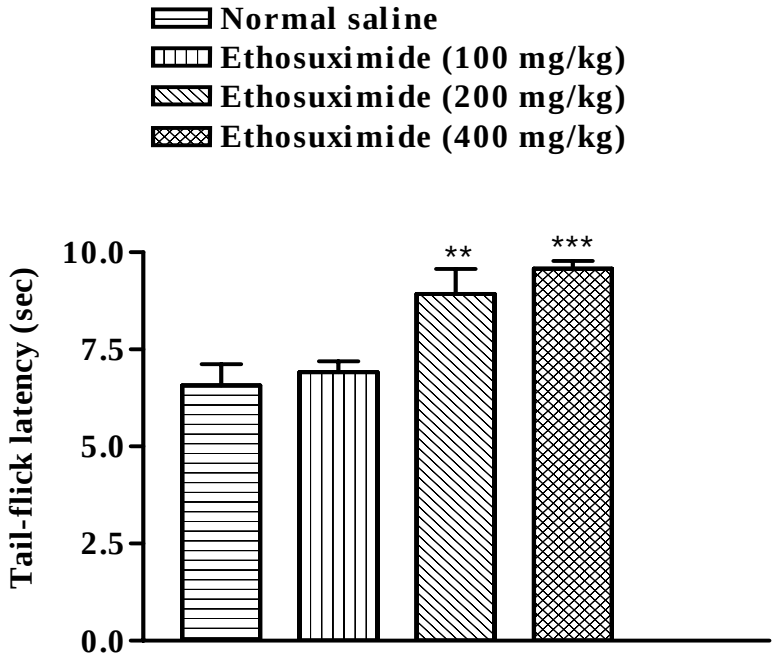


Figure 6. Effect of ethosuximide on development of morphine tolerance in mice. The animals received morphine (20 mg/kg 2time/day for 4 days) for induction of morphine tolerance and simultaneously with each dose of morphine received ethosuximide (100, 200 or 400 mg/kg i.p.). On the next day, animals received morphine (7 mg/kg) and tail-flick test were carried out to evaluate the Effect of ethosuximide on development of morphine tolerance. Each group comprised 7 mice. Data are means + S.E.M. **P < 0.01, ***P < 0.001 different from the saline control group (ANOVA followed by Tukey-kramer test).

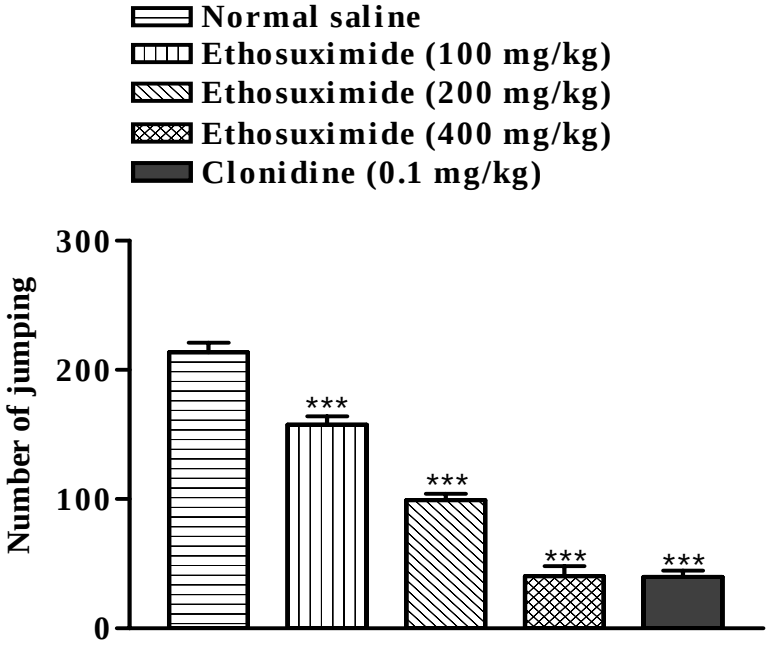


Figure 7. Effect of ethosuximide on expression of morphine dependence in mice. The animals received morphine (50, 50 and 75 mg/kg /day for 3 days) for induction of morphine dependence. On day 4, animals received ethosuximide (100, 200 and 400 mg/kg), clonidine (0.1 mg/kg) or saline and 1.5 hour later Naloxone (5 mg/kg) was used, to test jumping. Each group comprised 7 mice. Data are means + S.E.M. ***P < 0.001 different from the saline control group (ANOVA followed by Tukey-kramer test).

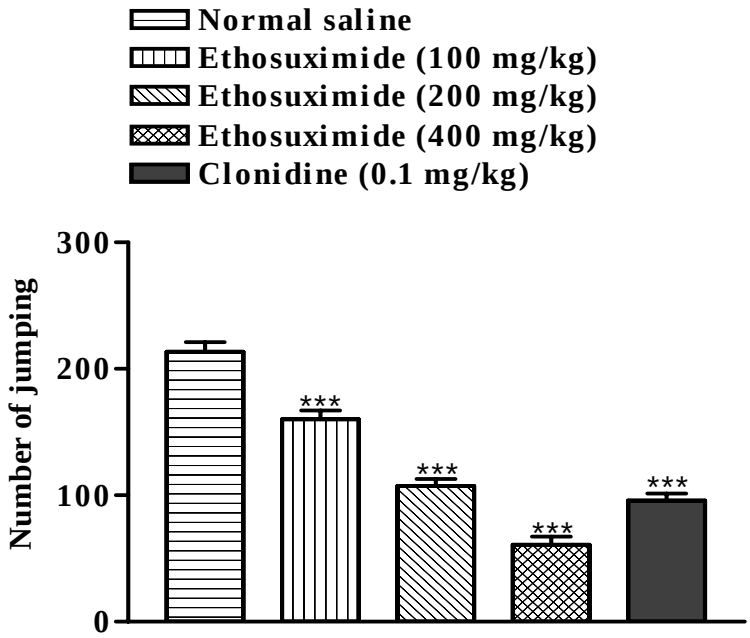


Figure 8. Effect of ethosuximide on development of morphine dependence in mice. The animals received (50, 50 and 75 mg/kg /day for 3 days) for induction of morphine dependence and simultaneously with each dose of morphine received ethosuximide (100, 200 and 400 mg/kg), clonidine (0.1 mg/kg) or saline. Naloxone (5 mg/kg) was used on day 4, to test jumping. Each group comprised 7 mice. Data are means + S.E.M. ***P < 0.001 different from the saline control group (ANOVA followed by Tukey-kramer test).

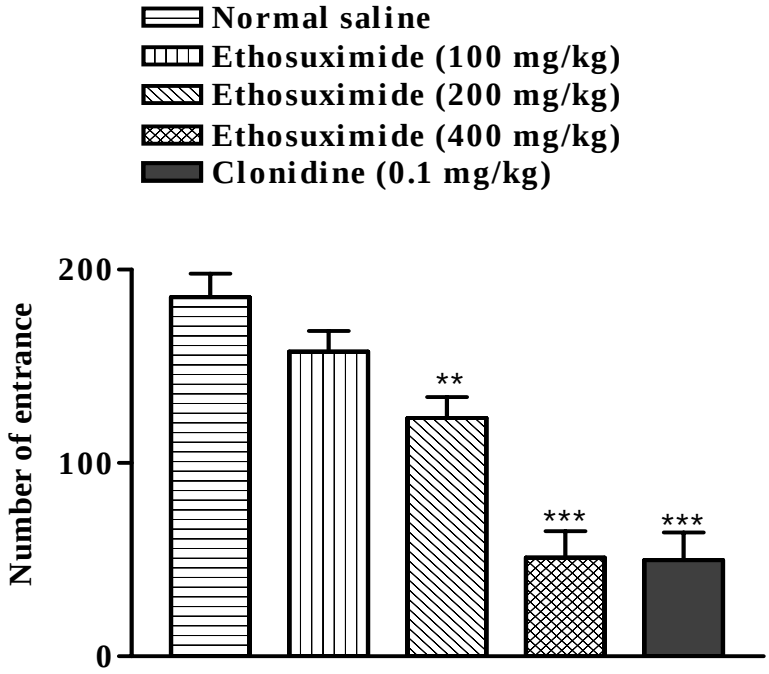


Figure 9. Effect of single dose of ethosuximide on locomotion in mice. The animals received morphine (50, 50 and 75 mg/kg /day for 3 days) for induction of morphine dependence. On day 4, animal received ethosuximide (100, 200 and 400 mg/kg), clonidine (0.1 mg/kg) or saline and before injection of naloxone, locomotor activity recorded for 10 min using open field apparatus. Each group comprised 7 mice. Data are means + S.E.M. **P < 0.01, ***P < 0.001 different from the saline control group (ANOVA followed by Tukey-kramer test).

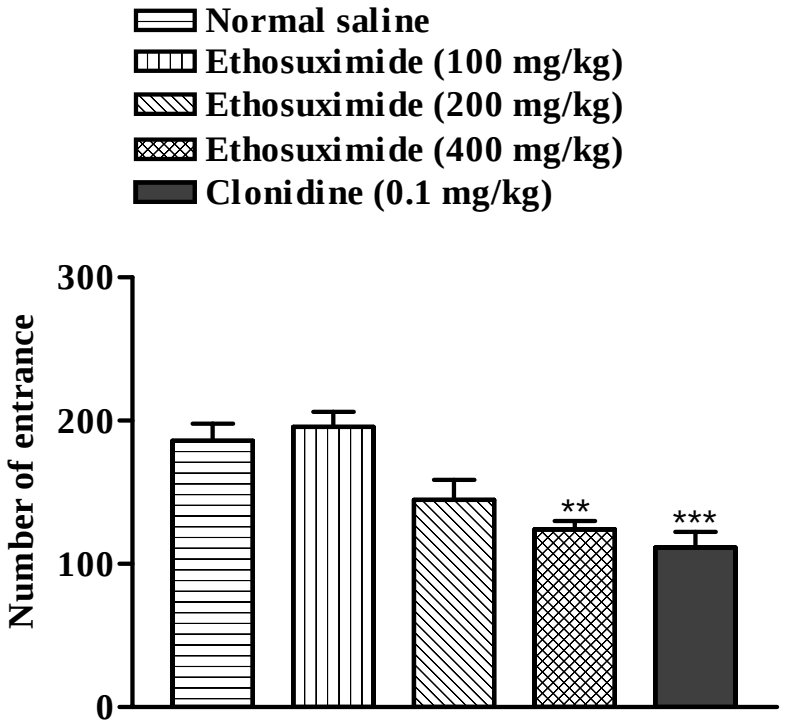


Figure 10. Effect of repeated doses of ethosuximide on locomotion in mice. The animals received morphine (50, 50 and 75 mg/kg /day for 3 days) for induction of morphine dependence and simultaneously with each dose of morphine received ethosuximide (100, 200 and 400 mg/kg), clonidine (0.1 mg/kg) or saline. On day 4, before injection of naloxone, locomotor activity recorded for 10 min using open field apparatus. Each group comprised 7 mice. Data are means + S.E.M. **P < 0.01, ***P < 0.001 different from the saline control group (ANOVA followed by Tukey-kramer test).

3.3. Effect of ethosuximide on the expression or development of naloxone-induced jumping behavior in morphine-dependent mice

Fig. 7 shows the effect of ethosuximide on the expression of naloxone-induced jumping. Single dose administration of ethosuximide (100, 200 or 400 mg/kg), 30 minutes before naloxone, significantly attenuated jumping in morphine-dependent animals. The maximum effect of ethosuximide was obtained with 400 mg/kg which was comparable with clonidine (0.1 mg/kg).

Effect of ethosuximide on the development of dependence has been showed in Fig. 8. Repeated administration of ethosuximide (100, 200 or 400 mg/kg, simultaneously with each dose of morphine, for 3 days), significantly decreased naloxone-induced jumping in morphine-dependent animals. The maximum effect of ethosuximide was obtained with 400 mg/kg which was more than clonidine (0.1 mg/kg).

3.4. Effects of ethosuximide on locomotion

Fig. 9 shows the effect of single administration of ethosuximide on locomotor activity. Different doses of ethosuximide (100, 200 and 400 mg/kg) decreased locomotion (statistically significant in doses 200 and 400 mg/kg). The effect of ethosuximide in dose 400 mg/kg was comparable with clonidine (0.1 mg/kg).

The effect of repeated doses of ethosuximide on locomotor activity has been showed in figure 10. Ethosuximide significantly decreased locomotion only in the highest dose (400 mg/kg). The efficacy of ethosuximide (400 mg/kg) was comparable with clonidine (0.1 mg/kg).

Discussion

The results of the present investigation demonstrate that ethosuximide attenuates development and expression of tolerance to antinociceptive effect of morphine. Furthermore ethosuximide has antinociceptive effect by itself that isn't antagonized by naloxone. Ethosuximide also decreases development and expression of naloxone-induced jumping in morphine-dependent animals. Finally, this study further suggests that T-type VDCC blockers augments the antinociceptive effects of morphine, prevents the development of tolerance to antinociceptive effects of morphine and suppress morphine withdrawal syndrome.

In this study we showed that ethosuximide has antinociceptive effect. The response to the drug was dose-dependent. To clarify the probable role of opioid receptors or endogenous opioid system in this effect, we tested antinociceptive effect of ethosuximide in the presence of naloxone. The antinociception completely remained and therefore this mechanism was excluded. Previously Matthews and Dickenson showed that spinal administration of ethosuximide (5–1055 μg) mediated significant inhibition of the electrical and natural (innocuous and noxious) evoked rat dorsal horn neuronal responses after spinal nerve ligation and suggested some role for T-type Ca^{2+} channels in this effects of ethosuximide (14). Also Dogrul (2002) suggested that T-type Ca^{2+} channel blocker augments the antinociceptive effects of morphine (5). They suggest that activation of opioid mu receptors inhibit T-type VDCCs and this opioid-induced inhibition of calcium currents diminishes calcium-dependent neurotransmitter release from presynaptic terminals and therefore, potentiate analgesia. Other study showed when injected in peripheral receptive fields of sensory neurons, ethosuximide prolongs the paw withdrawal latency and it was suggested that the blockade of T-type Ca^{2+} channels in sensory neurons by ethosuximide could contribute to attenuation of thermal nociception (19).

The present results also showed ethosuximide attenuates the development of tolerance to antinociceptive effect of morphine. The response to the drug was dose-dependent. For testing this effect of ethosuximide, we administered the drug for 4 days simultaneously with morphine and on next day we carried out tail-flick test and compared the result with the result of morphine plus saline. Considering antinociceptive effect of ethosuximide by itself, one may claim that ethosuximide reduces the tolerance to morphine because it remained until next day and exerts direct antinociceptive activity. For clarifying this doubt, in other part of study we administered the maximum dose of ethosuximide (400 mg/kg, 2time/day) for 4 days and on the next they we did tail-flick test on these animals. The results showed lack of any antinociceptive effect. Therefore it concludes that the effect of ethosuximide in attenuating the development of tolerance to antinociceptive effect of morphine is not related to its remained direct antinociceptive effect. Ethosuximide also showed inhibitory effect on the expression of tolerance to antinociceptive effect of morphine. For evaluation of this effect of ethosuximide, we administered morphine for 4 days to induce tolerance and on next day, morphine and ethosuximide were administered and after doing tail-flick test the result were compared with the result of morphine plus saline. Considering direct antinociceptive effect of ethosuximide in doses 200 and 400mg /kg that interfere with antinociceptive effect of morphine, it was possible to do this part of study only by dose 100 mg/ kg that doesn't have direct analgesic effect.

The prevention of tolerance by ethosuximide may be related to its capacity to block T-type VDCCs. The upregulation of VDCCs has been demonstrated for L and N-type VDCCs following chronic morphine administration (20, 21) but not evaluated for T-type VDCCs. Mibefradile, a T-type calcium channel blocker, reduces tolerance to antinociceptive effect of morphine (5).

Different doses of ethosuximide, when administered 30 min before naloxone in morphine-dependent mice, decreased naloxone-induced jumping. The response to the drug was dose-dependent. The data may indicate that ethosuximide influences the expression of morphine dependence. Repeated administration of ethosuximide simultaneously with each dose of morphine also reduced naloxone-induced jumping. The response to the drug was dose-dependent. The data may indicate that ethosuximide decreases the development of morphine dependence. The effect of maximum dose of ethosuximide (400 mg/kg) in the expression and development of morphine dependence is comparable with clonidine (0.1 mg/kg) as positive control of study. The results of locomotion test showed that in single dose study ethosuximide (in doses 200 and 400 mg/ kg) and clonidine (0.1 mg/kg) reduce the activity of animals. In repeated dose study, ethosuximide (only in 400 mg/ kg) and clonidine (0.1 mg/kg) reduce the activity of animals but less than single dose study. Therefore it can conclude that reduction of locomotion may be one of the reasons to explain the effect of ethosuximide and clonidine in decreasing the expression and development of morphine dependence. But regarding the significant reduction exerted by lower doses of ethosuximide, (that don't have significant effect on locomotor activity) it can conclude that other mechanism also may be involved. It has been hypothesized that dependence develops as adaptive mechanisms which promote increased calcium influx into neurons as a means of counteracting the effects of opioids (5). Regarding this hypothesis it is explicable that calcium channel blockers can attenuate tolerance. It has been shown that L-type VDCC blockers attenuates the behavioral signs of morphine withdrawal syndrome (22, 23). Mibefradil, as a T-type calcium channel blocker, also prevents the expression of the abstinence syndrome when given directly before administration of naloxone. This indicates an involvement of T-type VDCCs in the acute abstinence syndrome.

These results showed that ethosuximide, a relatively selective T-type VDCC blocker has direct antinociceptive effect, prevents the development of antinociceptive tolerance to morphine and suppress morphine withdrawal syndrome. These results suggest ethosuximide modify the effects of morphine in a manner that may be clinically useful to enhance morphine analgesia, to prevent antinociceptive tolerance and to attenuate the acute morphine withdrawal syndrome.

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