



Verapamil Interferes With the Anticonvulsant Effect of Morphine in A Strychnine Induced Convulsion Model in Mice

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ABSTRACT

Background: Opioids exert different effects on seizure threshold based on their doses and the models. P-glycoprotein (p-gp) prevents various substances from entering the brain. Morphine is a p-gp substrate. The aim of this study was to investigate the effects of morphine on strychnine-induced convulsion in ovariectomized mice and to see whether verapamil as a p-gp pump inhibitor interferes with that effects. **Methods:** Female mice were ovariectomized to remove the gonadal sex hormones. Thirty minutes prior to induction of convulsion by subcutaneous injection of strychnine, the animals were received morphine (1, 2, 3mg/kg), naloxone (1, 2, 5mg/kg), verapamil (20mg/kg) and morphine (3mg/kg)+verapamil (20mg/kg) intraperitoneally. Then, the onset of convulsion and time to death were recorded. The significance of differences for the seizure parameters were assessed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc comparison or unpaired t-test. **Results:** The results showed that morphine produced anticonvulsant effect by prolonging the time to death ($P < 0.001$) and naloxone had no effect on strychnine-induced convulsion. Verapamil alone had no effect on the convulsion whereas, it significantly ($p < 0.05$) reduced the anticonvulsant effect of morphine by reducing the time to death. **Conclusion:** Anticonvulsant activity of morphine is mediated through different mechanisms. The reduced anticonvulsant effect of morphine by verapamil seems to be because of increase in morphine concentration in brain.

Introduction

Seizure is a common neurological disorder that occurs as a result of imbalance in excitatory and inhibitory pathways in central nervous system (CNS). Seizure-controlling structures are functionally connected to neuronal pathways influenced by the γ -aminobutyric acid (GABA) and excitatory amino acids (EAAs) neurotransmitter systems. These systems are important in maintaining the inhibition and excitation balance of neuronal function. When this balance is changed, seizures may occur. Opioids acts as neuromodulators that might affect the balance of neural excitation and inhibition, particularly in the seizure-controlling brain structures¹. Opiates seem to be involved in producing seizure activity within the hippocampus, or alternatively, that they may be released by seizure activity within the limbic system to prolong the period of postictal depression and thereby prevent the seizures recurrence during this period². It has been reported that low doses of morphine show an anticonvulsant effect against seizure models induced by GABA-transmission

blockers, picrotoxin, bicuculline, pentylenetetrazole (PTZ), isoniazid and higher doses of this opioid-receptor agonist enhance the susceptibility of animals to the same seizure models^{3,4}. Because of the lack of investigation on the effect of opioid on strychnine-induced convulsion, the purpose of the present study was to determine the effect of naloxone and morphine on strychnine-induced convulsion model in ovariectomized mice.

Pharmacotherapy of central nervous system diseases is limited by the blood-brain barrier (BBB)⁵. The BBB is composed of specialized endothelial cells that prevent diverse molecules from entering the brain. The BBB is known to exclude nearly all molecules from entering the brain except small or lipophilic molecules. This is important to protect the brain from exposure to toxic molecules and to retain the proper internal environment for brain function. Membrane proteins exist in brain capillaries. They transport lipophilic molecules that enter the endothelial cells back to the blood. P-gp is

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one such transporter that is an important component of the BBB⁶ and present in high concentration on apical surface of endothelial cells in brain capillaries^{7,5,6}. Here it transports substrates toward the blood. Thus this transporter can limit the penetration into and retention within the brain. Therefore it modulates effectiveness and central nervous system toxicity of numerous compounds⁷. It is an ATP-dependent transport protein that entangled in extruding a variety of structurally unrelated compounds and preventing their accumulation within the brain^{8,5,6}. The extended substrate spectrum explains the ability of P-glycoprotein to provide cross-resistance to multiple classes of chemotherapeutic agents⁵. P-gp prevents the entry of various drugs that are used in the treatment of central nervous system disorders⁶. It also limits a large number of prescribed drugs entering into the brain and contributes the poor success of CNS drug⁵. Modulation of P-gp can affect drug bioavailability, increase or decrease entering of its substrates into the brain, and affect the therapeutic efficacy⁸.

The calcium-channel blocker verapamil is a Pglycoprotein inhibitor^{6,9,7} and can function to block P-glycoprotein-modulated efflux of antiepileptic drugs in the brain¹⁰. Since Modulating P-glycoprotein can improve p-gp substrate drug delivery into the brain^{8,5} and morphine is a p-gp substrate⁸, another purpose of the present study was to assess the interaction of morphine with p-gp inhibitor verapamil on convulsion in this model.

Materials and Methods

Drugs

Strychnine, verapamil/lekoptin, morphine sulfate, Naloxone hydrochloride, Ketamin and Xylazine were used. Strychnine was purchased from Sigma Chemical Co (USA). Morphine sulfate was obtained from Iran TEMAD Co (Tehran, Iran). Naloxone hydrochloride was purchased from Iran Tolid Daru Co, (Tehran, Iran). Verapamil was obtained from Drugpro, Pharmaceuticals (USA). Ketamin (1/10) and Xylazine (1/2) purchased from Alfasan Pharmaceuticals Holland.

Morphine sulfate dissolved in physiological saline solution and 5 ml/kg of it was injected to mouse body weight. Strychnine was prepared in physiological saline and 5 ml/kg of it was injected to mouse body weight. Fresh solutions were made each day of drug testing. All drugs were administered intraperitoneally (i.p.) except Strychnine was given subcutaneously (s.c.).

Animals

Adult female albino BALB/c mice (Laboratory animal center, Jondishpour medical university, Ahvaz, Iran) aged 6–8 weeks and weighing 20–35 g were used in this study. Animals were housed under standard laboratory conditions that included controlled ambient temperature (21 ± 2°C), a 12-hour-dark/12-hour-light (7:00 a.m. to 7:00 p.m.) cycle, and free access to food (standard mouse chow pellets) and water except for the

short time that animals were removed from their cages for testing.

The animals were housed in groups of 4–5 per cage. The animals were randomly distributed into different groups. Each mouse was used only once, and by average each treatment group consisted of seven animals. All experiments were performed between 12:00 and 19:00 h.

All the procedures were carried out in accordance with institutional guidelines for animal care and use.

Ovariectomy

For the lack of effect of cyclic change of steroid hormones, the female mice were ovariectomized.

Ketamine (70 mg/kg) and xylazine (7 mg/kg) (11) were initially injected i.p. to animals until loss of consciousness and loss of any response. After abdominal incision, the ovary was then cut away from the uterus, and the uterus was allowed to settle back into the abdominal cavity. Subsequently, the skin was closed. Animals were placed in their home cage until recovery a few hours later. All subjects were allowed 14 days to recovery before experiments commenced.

Seizure determination

Our protocol for testing the anticonvulsant effects of drugs followed the procedures described by Yamaguchi and Rogawski (1999)¹². Animals were observed for a 30-min period after injection of chemical convulsant¹³. The onset of convulsion and time of death were measured after strychnine administration¹². Then drugs were evaluated for protective activity against strychnine-induced convulsion.

Experiments

In the first series of experiments, the effects of different doses of morphine and naloxone on strychnine induced convulsion were determined. Morphine (1, 2 or 3 mg/kg), naloxone (1, 2 or 5 mg/kg) or vehicle (saline) was administered i.p. to different groups. Then these groups received a s.c. injection of strychnine (1.2 mg/kg¹⁴) 30 min later. The time intervals for opioid injection were chosen based on previous published works^{15,16}. The doses of morphine^{13,4,17} and naloxone^{18,19,20} were chosen based on previously published studies. In the second series of experiments, the effects of verapamil and interaction of it with opioids were examined. In one group verapamil (20 mg/kg)^{21,9,22} were administered i.p. 60 min²³ before the s.c. injection of strychnine (1.2 mg/kg). The interaction of the verapamil and the anticonvulsant effect of morphine was studied in pretreatment groups. In pretreatment experiment, we injected verapamil (20 mg/kg ip) to the mice 60 min before effective dose of morphine (3 mg/kg) and strychnine injected subcutaneously 30 min later.

In all groups, for a 30 min period after injection of strychnine, onset of convulsion and time of death were measured.

Data analysis

Data are presented as mean ± S.E.M. The significance of differences for the seizure parameters between control and morphine or naloxone received group were assessed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc comparison.

The significance of differences for the seizure parameters between morphine alone and morphine in the presence of verapamil -treated group were assessed

using unpaired *t*-test. The significance level was defined as $P < 0.05$.

Results

Figure 1 illustrates the effect of different doses of morphine (1, 2, 3 mg/kg) on onset of convulsion and death time in strychnine-induced convulsion in OVX mice. Compared to the vehicle-treated group, Morphine significantly increased death time ($P < 0.001$) when compared to vehicle.

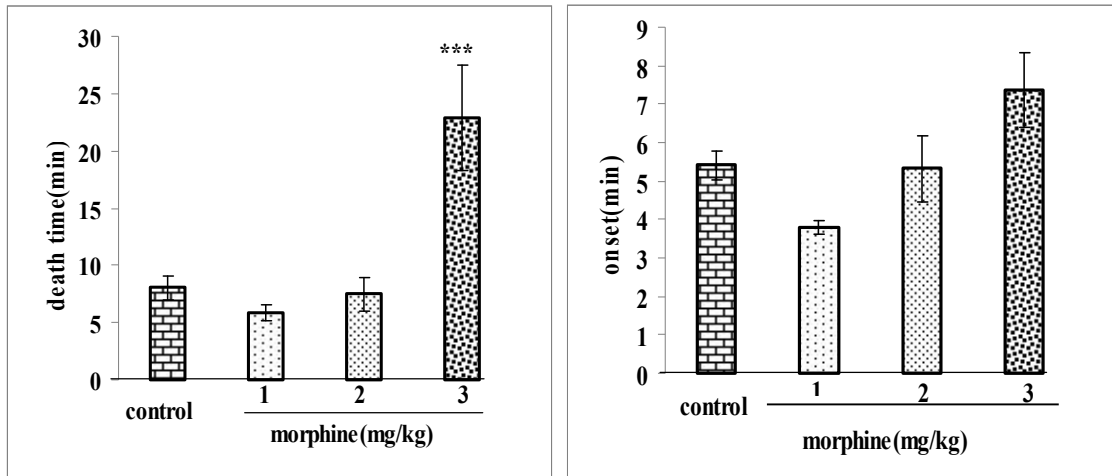


Figure 1. Effect of morphine on onset and death time in strychnine-induced convulsion in mice. Morphine (1, 2, 3 mg/kg) or saline were administered intraperitoneally and strychnine was administered 30 min later. Then seizure parameters evaluated for 30 min period. Data represent means ± SEM of seven mice. *** $P < 0.001$ compared with vehicle-treated group (shown as control).

Figure 2 illustrates the effect of naloxone (1, 2, 5mg/kg) on onset of convulsion and death time in strychnine-induced convulsion in OVX mice compared

to the vehicle-treated controls. One-way ANOVA analysis showed no difference between naloxone and vehicle -treated group in seizure parameters.

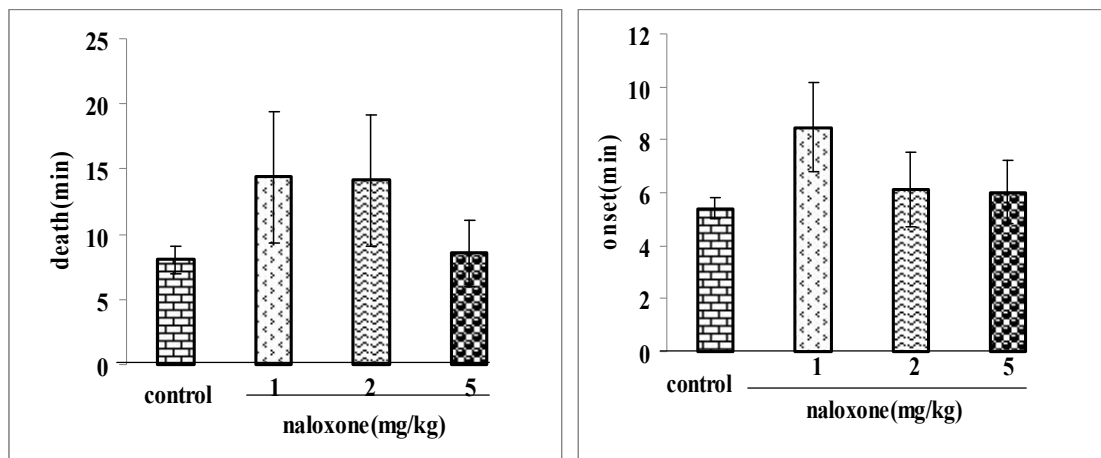


Figure 2. Effect of naloxone (1, 2, 5mg/kg) on onset and death time in strychnine-induced convulsion in mice. Naloxone (1, 2, 5mg/kg) were administered intraperitoneally and strychnine was administered 30 min later. Control group was received saline and strychnine was administered 30 min later. Then for 30 min period seizure parameters evaluated. Data represent means ± SEM of seven mice

Figure 3 illustrates the effect of verapamil (20 mg/kg) on strychnine-induced convulsion in OVX mice compared to the vehicle-treated controls. Unpaired *t*-

test analysis showed no difference between verapamil and vehicle -treated in seizure parameters.

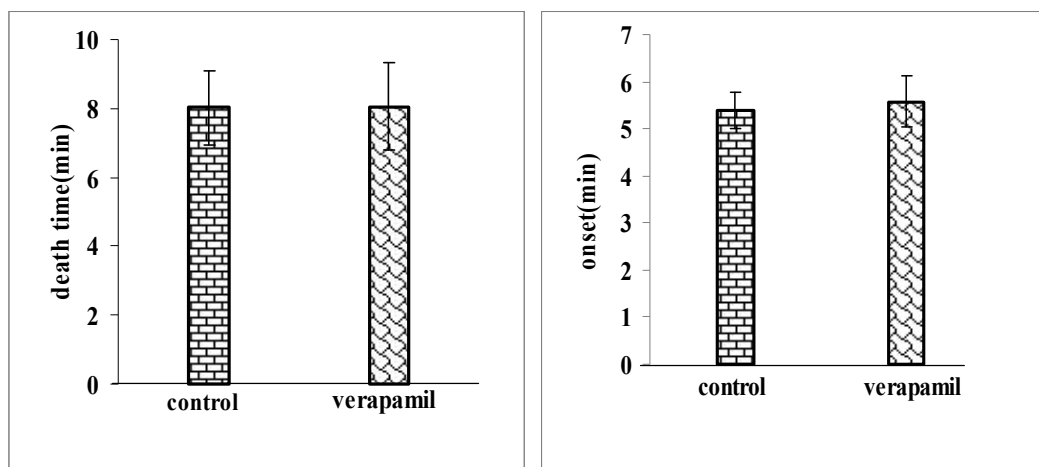


Figure 3. Effect of verapamil on onset and death time strychnine-induced convulsion in mice. Verapamil (20 mg/kg) were administered intraperitoneally and strychnine was administered 60 min later. Control group was received saline then seizure parameters evaluated for 30 min period. Data represent means \pm SEM of seven mice.

Figure 4 illustrates the effect of pretreatment of verapamil (20 mg/kg ip) with morphine (3 mg/kg ip) on the anticonvulsant property of morphine in strychnine-induced convulsion in OVX mic. Unpaired *t*-test analysis showed that verapamil decreased the death

time ($P < 0.05$) of convulsion in morphine-treated group significantly. Thus the combined treatment of verapamil with morphine reduced anticonvulsant effect of morphine.

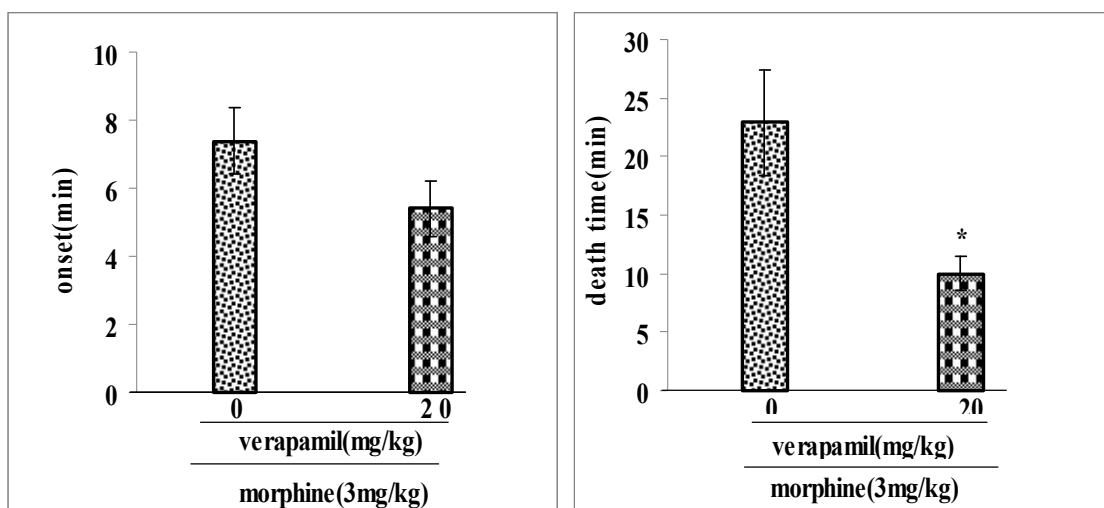


Figure 4. Effect of pretreatment verapamil with morphine on onset of convulsion and death time in strychnine-induced convulsion in mice. Animal were received morphine (3 mg/kg ip) or verapamil (20 mg/kg ip) + morphine (3 mg/kg ip, 60 min later injection of verapamil) and received strychnine 30 min later. Then seizure parameters evaluated for 30 min. Data represent means \pm SEM of seven mice. $P < 0.05$ compared with morphine-treated group (shown as 0).

Discussion

The most important finding of the present study is that the morphine (1, 2, 3mg/kg) had anticonvulsant effect on convulsion induced by subcutaneous injection of strychnine in OVX mice dose dependently (by its ability to prolong the death time). Naloxone (1, 2, 5mg/kg) had no effect on strychnine-induced convulsion in OVX mice that is in line report of Dingledine and co werker (1997)²⁴. In this study verapamil (20 mg/kg) had no effect on strychnine-

induced convulsion in OVX mice, whereas it reduced anticonvulsant effect of morphine.

The anticonvulsant effect of opioids is suspected to be mediated through inhibitory G proteins coupled receptors of opioid peptides^{17,13}. These receptors exert their various effects by activating guanosine triphosphate-binding protein and their effectors such as adenylate cyclase and ion channels^{25,13,26,27}. Opioids show a wide range of their effects by coupling to such inhibitory Gi/Go proteins^{28,13,17}, leading to reduce neuronal cyclic AMP levels, reduce Ca⁺⁺

conductance^{13,28}, shortening of action potential duration (APD) and decrease neurotransmitter release^{25,13}. However, some reports indicate that opioids may also have direct stimulatory effects on intracellular signaling mechanisms including stimulation of adenylyl cyclase, increasing calcium influx, prolongation of APD and increasing neuronal excitability¹³.

It is suggested that GABA and glutamate modulation can be a predominant mode of opioid action^{29,30}. Kappa opioid receptor activation modulates glutamate-induced excitatory synaptic transmission in the CNS and can decrease NMDA-induced brain injury³¹. Delta opioid agonists have been reported to reduce both inhibitory and excitatory neurotransmission and while reduced GABA_A receptor-induced inhibition would exacerbate seizures, decrease in excitatory activity should inhibit seizures³². Morphine, by acting on μ receptors, inhibits release of various neurotransmitters²⁷, modulates GABA- and glutamate-induced responses³³ and it appears that GABAergic systems have particular significance for the elucidation of the various effects of morphine on seizure susceptibility³⁴. It is possible that the mechanism of anticonvulsant action of morphine is mediated by enhancing GABAergic activity^{35,17,13,3,19} and inhibitory effects on brain excitatory pathways (such as glutamatergic pathways)^{35,17,13}. Presynaptic inhibitory effect of morphine is likely induced by the reduction of Ca²⁺ entering into nerve terminals and thereby prevents the release of glutamate in the cerebral cortex³⁶. Opiate depresses the stimulated release of the excitatory transmitter by decreasing the supply of Ca²⁺ ions to the stimulus-release coupling mechanism in the sympathetic nerve terminals³⁷.

It is possible that the activation of NO system by different doses of morphine can lead to opposite effects on seizure susceptibility and NO might induce anticonvulsant effect of morphine by an increase in GABAergic tone⁴.

In our study verapamil (20 mg /kg, i.p) had no effect on seizures produced by subcutaneous strychnine in ovariectomized mice. In contrast verapamil (5-20 mg /kg, i.p) exhibited anticonvulsant activity in the study of Umukoro and co werker (2006) by their ability to prolong the onset of seizures produced by intravenous strychnine (1 mg kg⁻¹) in mice²². Thus mechanism of epileptogenises of interavenous strychnine is different from subcutaneous model.

In the present study pretreatment of this dose of verapamil with morphine decreased anticonvulsant effect of morphine in strychnine- induced convulsion. Several reports exist that opioid receptor agonists can affect seizure susceptibility in a biphasic manner causing dose-dependent anti- and pro-convulsant effect, respectively, with increase in its concentration^{13,3,38}. Acute administration of morphine exerts a biphasic pattern depending on the doses used. While low doses of morphine show an anticonvulsant effect against seizure models induced by GABA-transmission blockers, picrotoxin, bicuculline,

pentylene-tetrazole (PTZ)^{39,4,17,35,40}, isoniazid³, and kainic acid⁴¹. Higher doses of it increase the susceptibility of animals to the same seizure models^{3,4,17,35,40}. Thus the increase in morphine concentration in brain subsequent to inhibition of P-gp by verapamil have been proposed as a probable mechanism that mediate verapamil-induced proconvulsant in pretreatment group.

Also Verapamil is an active analogue of papaverine, an alkaloid found in the opium poppy. Verapamil has agonistic activity on μ , δ and κ_3 receptor subtypes and increased the analgesic effect of opiates in various behavioral tests³⁶. Thus it is probable that agonistic activity of verapamil on opioid receptor may increase the effect of morphine. Beside it increase the concentration of morphine, so increase the excitotoxic activity of high concentration of morphine in brain.

Conclusion

In conclusion, the present study showed that morphine has anticonvulsant effect and naloxon had no effect on strychnine- induced convulsion. Verapamil had no effect on convulsion itself but it reduced anticonvulsant effect of morphine in this model. Anticonvulsant activity of morphine can be induced through different mechanisms. The reduced anticonvulsant effect of morphine by verapamil seem to be as a result of increase in morphine concentration in brain, but it should be keep in mind that another unknown mechanism can play a role.

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