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Original Article

Antinociceptive effect of clavulanic acid and its preventive activity against development of morphine tolerance and dependence in animal models

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Abstract

Glutamate has a key role in pain perception and also development of tolerance and dependence to morphine. It has been reported that clavulanic acid affects glutamatergic transmission via activation of glutamate transporter. Therefore the present study was aimed to evaluate the possible antinociceptive effect of clavulanic acid and its preventive activity against development of morphine tolerance and dependence in animal models. Male Swiss mice (25-30 g) were used in this study. Acetic acid-induced writhing, formalin test and hot plate method were used to assess the antinociceptive effect of clavulanic acid. Morphine (30 mg/kg, s.c.) was administered to the mice two times a day (8 AM and 4 PM) for 3 days in order to produce tolerance. To develop morphine dependence, morphine sulfate (50, 50 and 75 mg/kg) was injected at 8 and 12 AM and 16 PM respectively and for 3 consecutive days. Naloxone (5 mg/kg, i.p) was used to induce morphine withdrawal syndrome and the number of jumps and presence of ptosis, piloerection, tremor, sniffing and diarrhea were recorded and compared with control group. Clavulanic acid at doses of 10, 20 and 40 mg/kg inhibited abdominal constriction and licking behavior of acetic acid and formalin-induced pain respectively. Clavulanic acid was not able to show any antinociception in hot plate model and could not prevent development of tolerance and dependence to morphine. Clavulanic acid has considerable antinociceptive activity and further studies are needed to clarify its exact mechanism.

Keywords: Clavulanic acid; Antinociceptive; Tolerance; Dependence; Morphine

INTRODUCTION

Nowadays addiction to opioids is a critical issue in many countries. Methadone and buprenorphine maintenance treatments are the most popular methods for opioid detoxification. Since these are opioid drugs, they can also induce dependence after prolonged use and therefore researchers are looking for a novel replacement to alleviate morphine withdrawal syndrome (1-3).

Many neurotransmitter systems including dopaminergic (4,5), adrenergic (6), purinergic (7), excitatory amino acids (8-10) and nitric oxide (11) have been tested on opioid tolerance and dependence. In recent years, researchers have focused on the role of glutamate in this issue. It has been reported that activation of N-methyl-D-aspartate (NMDA) receptors as an important class of glutamate receptors has a key role in the development and expression of opioid dependence and tolerance and NMDA receptor antagonists have shown beneficial effects in the suppression of morphine withdrawal syndrome (12-14).

Glutamate transporter subtype 1 (GLT1) has an important role in cellular uptake of glutamate in the brain and termination of glutamatergic transmission. A large number of clinically approved drugs have ben screened for their potential activation of GLT1 and reported that β-lactam antibiotics can increase glutamate uptake through GLT1 activation (15). Later it was also shown thstceftriaxone as a β-lactam antibiotic inhibits development of morphine physical dependence in rats (16). Also in a recent study it was reported

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that ceftriaxone alleviates neuropathic pain in the rats (17).

Clavulanic acid is structurally similar to penicillins and cephalosporins but unlike them has a weak antibacterial activity. It acts as an inhibitor of bacterial beta-lactamases and is administered in combination with some beta-lactam antibiotics like amoxicillin and ticarcillin, to potentiate and extend their antimicrobial activity (18). It has been reported that clavulanic acid also activates GLT1 (15,19). It has considerable oral availability (64-75%) and good penetration into the brain with a CSF/plasma ratio around 0.25 (20-22). Therefore, in this study clavulanic acid was selected as a candidate to be evaluated for possible antinociceptive effect and also preventive activity against development of morphine tolerance and dependence in animal models.

MATERIALS AND METHODS

Experimental animals

Male Swiss mice weighing 25-30 g were obtained from the animal house of the School of Pharmacy, Isfahan University of Medical Sciences, Iran.

Animals were housed in standard laboratory conditions throughout the study. They were placed in polypropylene cages with free access to water and food. All protocols were performed according to the guidelines for the laboratory care of animals approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran.

Chemicals

All of the chemicals and reagents were of analytical grade. Acetic acid and formalin were purchased from Merck Chemical Company (Germany). Morphine (Darou Pakhsh Pharmaceutical Co., Iran), clavulanic acid as potassium clavulanate (Farabi Pharmaceutical Co., Iran) and indomethacin (Sigma, USA) were also used.

Acetic acid writhing test

Male Swiss mice (25-30 g) were divided into 5 groups of 6 mice each. Control group received normal saline (10 ml/kg; intra-peritoneal (i.p.)) and the test groups were treated with 10, 20 or 40 mg/kg, i.p. of clavulanic acid. The fifth group received indomethacin as the reference drug at a dose of 10 mg/kg, i.p.

Abdominal contractions were induced in mice by i.p. injection of 1% (v/v) solution of acetic acid (10 ml/kg, i.p.). Vehicle, clavulanic acid and indomethacin were injected 30 min prior to acetic acid injection. After administration of acetic acid, the animals were put in cages and the number of abdominal twitches were counted within a 10 min period started 10 min after i.p. injection of acetic acid (23,24).

Formalin test

Mice were divided into 5 groups each containing 6 mice. The control group received normal saline (10 ml/kg; i.p.) and the test groups were treated with 10, 20 and 40 mg/kg of clavulanic acid (i.p.). Reference group received morphine (10 mg/kg, s.c.). Thirty min later, 20 µl of a dilute solution of formalin (2.5% v/v) was injected into dorsal surface of the right hind paw. Then the mice were moved to an observation chamber with a mirror at its bottom to make the observation of paw licking easier.

Two distinct periods of licking activity were identified: The early response and the late one that were recorded during 0-5 and 20-30 min respectively (24,25).

The pain during the early and late periods was indicative of acute and chronic (inflammatory) pain respectively.

Hot plate test

Male Swiss mice (25-30 g) were divided into 4 groups (6 animals in each group). Control animals received normal saline (10 ml/kg, i.p.), while the standard group received morphine (10 mg/kg, i.p.) and the test groups received clavulanic acid (50 and 100 mg/kg, i.p.). Animals were individually placed on a thermostatically controlled hot-plate (Borj Sanat, Iran) maintained at 58 ± 0.5 °C. The pain threshold was considered to be reached when the animals attempted to jump out of the plate. And the time of the first jump was recorded as the latency period. The cut-off

time was 15 s to avoid any tissue damage. Before drug administration, baseline latency was examined and animals with baseline latency lower than 3 s or above 30 s were excluded. After clavulanic acid or morphine injection, pain latency checked at 30 min intervals until 2 h (26). Percent of maximal possible antinociceptive effect (MPE%) was calculated using the following formula .

MPE% = [test latency (s)-control latency (s)]/[cut-off time (s)- control latency (s)] × 100

Development of tolerance to morphine antinociception

Morphine (30 mg/kg, s.c.) was administered to mice two times a day (8 AM and 4 PM) for 3 days in order to produce tolerance. Thirty min before each morphine injection, mice were pretreated with clavulanic acid (50 or 100 mg/kg, i.p.) or vehicle. On the day 4, antinociceptive tolerance to a challenge dose of morphine (5 mg/kg, s.c) was evaluated using the hot-plate test as described in the previous section (27).

Induction of morphine dependence in mice

To develop morphine dependence, three groups of mice (n=6) received morphine sulfate (25, 50 and 75 mg/kg) at 8 and 12 AM and 16 PM respectively and for 3 consecutive days. A dose of 50 mg/kg of morphine sulfate was also injected on the 4th day. Two groups of the animals also received clavulanic acid (50 and 100 mg/kg, i.p.) from second day and 30 min prior to morphine administrations. Control group was injected with saline (10 ml/kg, i.p.) instead of clavulanic acid. Naloxone (5 mg/kg, i.p.) was injected 2 h after the last morphine injection, and the number of jumps was counted immediately after naloxone injection over a 30 min period. Other withdrawal symptoms including ptosis, piloerection, diarrhea, sniffing and tremor were observed and scored over 3×10 min periods giving one point for the presence of each symptom in each 10 min period (maximum score = 3 points) (28).

Statistical analysis

Data were expressed as mean ± S.E.M. Statistical analysis were done with one-way analysis of variance (ANOVA) followed by Scheffe test using SPSS software Version 13.0. *P* values less than 0.05 were considered significant. To compare non-parametric data, Kruskall-wallis was followed by Mann-Whitney U test.

RESULTS

Acetic acid-induced writhing test

In acetic acid-induced writhing test, clavulanic acid at doses of 10 and 20 mg/kg significantly ($p<0.05$) inhibited acetic acid-induced abdominal constrictions and a highly significant decrease was seen at dose of 40 mg/kg ($p<0.001$). In this test indomethacin, as the reference drug at a dose of 10 mg/kg produced about 81.8% reduction of writhes (Fig. 1).

Formalin test

In acute phase of formalin test, clavulanic acid at all tested doses significantly ($p<0.001$) inhibited formalin-induced pain and morphine as a standard analgesic drug significantly ($p<0.001$) reduced pain behavior (Fig. 2). In chronic phase, clavulanic acid at doses of 20 and 40 mg/kg like morphine caused significant inhibition of formalin-induced licking behavior (Fig. 3).

Hot plate test

In hot plate test, morphine as the standard drug produced significant analgesia 30 min after injection which remained until 90 min. Clavulanic acid even at a dose of 100 mg/kg could not increase the reaction time (Fig. 4).

Tolerance test

As it is seen in Fig. 5, three days treatment of morphine induced tolerance as compared with the group received vehicle during these days. In morphine tolerant group, the antinociceptive activity of morphine was significantly ($p<0.05$) less than non-tolerant animals. Pretreatment of animals with clavulanic acid (50 or 100 mg/kg, i.p.) could not reverse the development of tolerance.

Dependence test

Treatment of dependent mice with naloxone induced withdrawal syndrome characterized with jumping, piloerection, tremor, diarrhea

and ptosis. Clavulanic acid (50 or 100 mg/kg, i.p.) did not exert any significant change in the number of jumpings (Fig. 6). In addition, pretreatment with clavulanic acid could not suppress other symptoms of withdrawal syndrome (Data not shown).

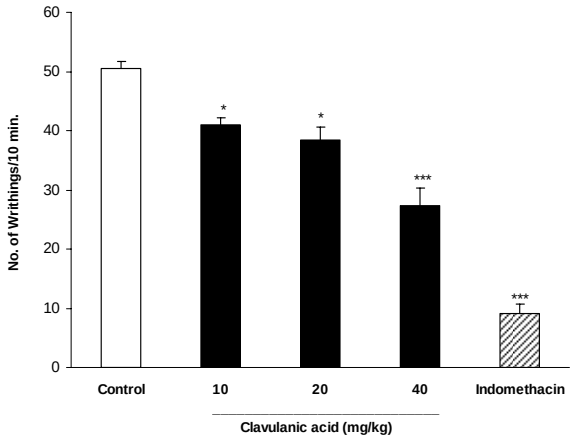

Fig. 1. Effect of i.p. injection of clavulanic acid on acetic acid-induced writhing test in mice (n=6). Clavulanic acid (10, 20 and 40 mg/kg), indomethacin (10 mg/kg) and the vehicle were administered 30 min prior to acetic acid (1%) injection (i.p.) and the number of abdominal contractions was counted for each mouse for a period of 10 min starting 10 min after acetic acid injection. The values represent the mean of abdominal twitches ± SEM. *p<0.05; ***p<0.001 compared with control group.

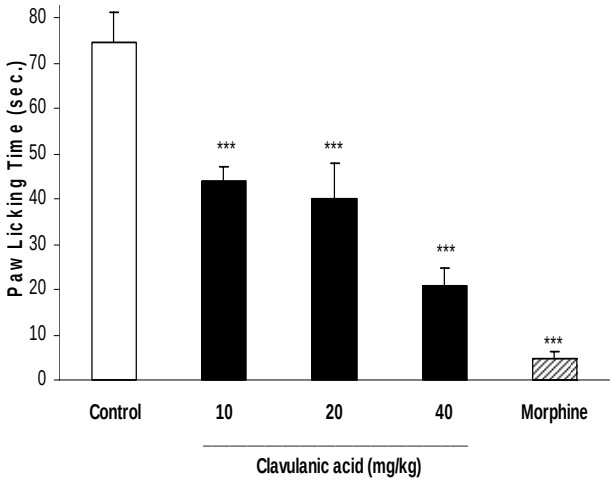

Fig. 2. The antinociceptive activity of clavulanic acid on hind paw licking during acute phase of formalin test. Different doses of clavulanic acid (10, 20, 40 mg/kg) and vehicle (normal saline) were administered i.p., 30 min prior to subplantar injection of formalin and time spent for licking was measured during a 0-5 min period started immediately after formalin injection. Morphine (10 mg/kg, i.p.) was used as reference drug. Data are mean ± SEM of 6 animals in each group. ***p<0.001 compared with control group.

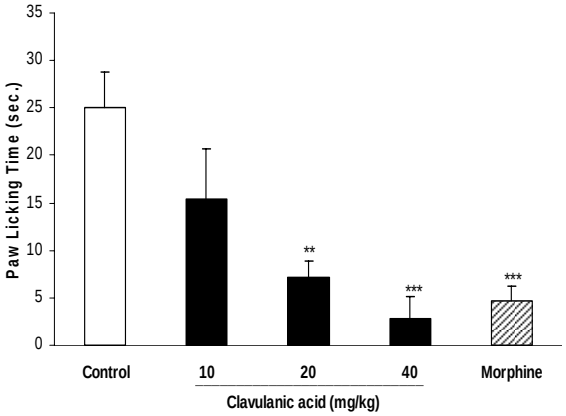

Fig. 3. The antinociceptive activity of clavulanic acid in chronic phase of formalin test. Different doses of clavulanic acid (10, 20, 40 mg/kg) and vehicle were administered i.p., 30 min prior to subplantar injection of formalin and time spent for licking was measured during a 20-30 min period after formalin injection. Morphine (10 mg/kg, i.p.) was used as reference drug. Data are mean ± SEM of 6 animals in each group. **p<0.01, ***p<0.001 compared with control group.

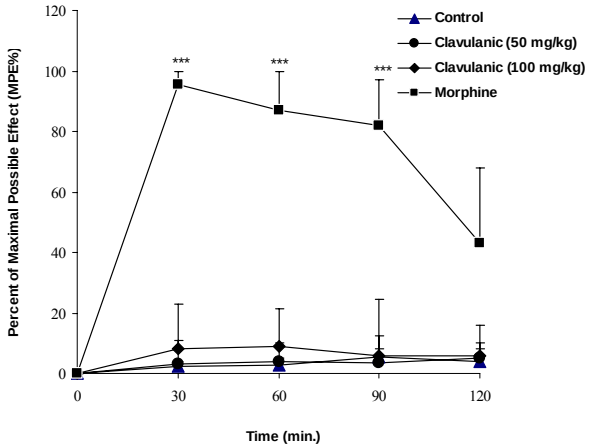

Fig. 4. The antinociceptive activity of clavulanic acid in hot plate test. Vehicle and clavulanic acid (50, 100 mg/kg, i.p.) were administered 30 min prior to placement of the animal in hot plate and reaction time of mice was measured at 30 min intervals until 2 h and percent of maximal possible antinociceptive effect (MPE%) was calculated for each time and compared. Morphine (10 mg/kg, i.p.) was used as reference drug. Data are mean ± SEM of 6 animals in each group. ***p<0.001 compared with control group.

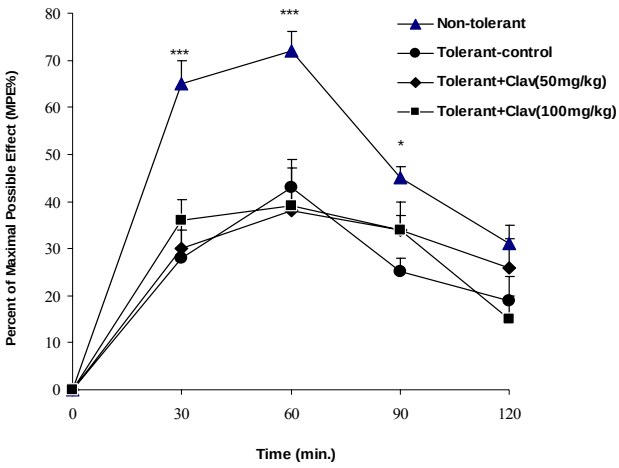

Fig. 5. Effect of clavulanic acid on development of tolerance to antinociceptive activity of morphine. Morphine (30 mg/kg, s.c.) was administered to mice two times a day (8 AM and 4 PM) for 3 days in order to produce tolerance. A group of animals (non-tolerant) received saline during these days. Thirty min before each morphine injection, mice were pretreated with clavulanic acid (50 or 100 mg/kg, i.p.) or vehicle. On day 4, antinociceptive tolerance to a challenge dose of morphine (5 mg/kg, s.c) was evaluated using the hot-plate test. Data are mean ± SEM of 6 animals in each group. *$p<0.05$, ***$p<0.001$ compared with control group.

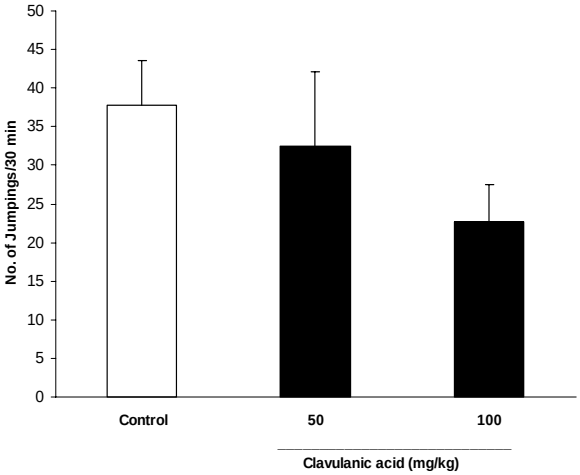

Fig. 6. Effect of clavulanic acid on naloxone-induced jumping behavior of morphine dependent mice. To develop morphine dependence, three groups of mice (n=6) received morphine sulfate (50, 50 and 75 mg/kg) at 8 and 12 AM and 16 PM respectively and for 3 consecutive days. A dose of 50 mg/ kg of morphine sulfate was also injected on the 4th day. Clavulanic acid (50 and 100 mg/kg, i.p.) was administered from second day and 30 min prior to morphine injections. Control group was injected with saline (10 ml/kg, i.p.) instead of clavulanic acid. Naloxone (5 mg/kg, i.p.) was injected 2 h after the last morphine injection, and the number of jumps was counted immediately after naloxone injection over a 30 min period. Data are mean ± SEM of 6 animals in each group.

DISCUSSION

The results of the present study indicated that clavulanic acid has good antinociceptive effect in acetic acid-induced writhing and formalin tests but showed no efficacy against thermal (hot plate) nociception. The formalin test is believed to represent a more valid model for clinical pain (29). In this test, the licking behavior of first (acute) phase (0-5 min) is believed to result from direct stimulation of nociceptive afferent fibers and the second or chronic phase (20-30 min) is due to an inflammatory process (30). It has been shown that drugs such as opioids which act mainly centrally, inhibit both phases of formalin-induced pain, while drugs, such as hydrocortisone and non steroidal anti-inflammatory drugs (NSAIDs) which are primarily peripherally acting, only inhibit the late phase (31,32). Clavulanic acid showed antinociceptive activity in both phases of formalin test and it means that at least a part of its effect is mediated centrally.

Co-administratin of clavulanic acid with morphine did not affect development of morphine tolerance and dependence. The development of morphine physical dependence and expression of somatic signs of morphine withdrawal are multifactorial processes which are mediated by several neural sites (e.g. locus coeruleus, hypothalamus) (33). Also several signaling pathways including adrenergic, dopaminergic, purinergic, excitatory aminoacids and nitric oxide are involved in the process of morphine dependence and tolerance (4-11). Recently studies have focused on the role of glutamate activity in morphine dependence and tolerance.

Two categories of glutamate receptors, NMDA and non-NMDA, and two distinct transporter systems mainly regulate glutamate activity (34). It has been shown that increased glutamate activity observed after repeated morphine administration mediates physical dependence and tolerance (35) and inhibitors of NMDA receptors have been shown to suppress morphine tolerance and dependence

(12-14). GLT1 is an astrocytic transporter which mediates 90% of cellular glutamate uptake in the brain and has a critical role in the termination of glutamatergic transmission (36). It has been reported that β-lactam antibiotics and in particular ceftriaxone activates GLT-1 (15). Based on these studies, Rawls and coworkers tested the effect of ceftriaxone on development of morphine physical dependence in rats and reported that this antibiotic significantly inhibits development of morphine physical dependence (16). In the present study we tested the effect of clavulanic acid as another GLT1 activator on the development of morphine physical dependence and tolerance. Unlike our expectation, clavulanic acid failed to inhibit development of morphine physical dependence and tolerance. It has been reported that clavulanic acid also affects dopamine transmission and enhances dopamine release (37). Also Kim and colleagues reported anxiolytic-like activity for this agent (38). In another study Rawl and coworkers reported anticonvulsant activity for clavulanic acid (19). Therefore it seems that clavulanic acid affects several neurotransmitters and these interactions may balance each other so that no significant inhibition of morphine physical dependence and tolerance is resulted. However this is just a scientific suggestion and needs further studies to clarify this issue.

CONCLUSION

It is concluded that although clavulanic acid can not prevent development of tolerance and dependence to morphine, it has considerable antinociceptive activity and further studies are needed to clarify its exact mechanism.

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