

Synergistic analgesic effect of morphine and tramadol in non-sensitized and morphine-sensitized mice: an isobolographic study

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Morphine and tramadol are the opioid analgesic drugs acting *via* activation of μ -opioid receptors. It is important to understand which mechanism (synergistic or additive anti-nociceptive activity) induced potent anti-nociceptive effect by co-administration of morphine and tramadol. Identification of new strategies that can potentiate analgesic effects of opioids will be good therapeutic approaches for pain relief. To this aim, male mice were cannulated in the left ventricle by a stereotaxic instrument. A tail-flick test was used to record the pain threshold. The results revealed that intracerebroventricularly injection of morphine induced an anti-nociceptive effect in non-sensitized and morphine-sensitized mice. We found that infusion of tramadol produced an anti-nociceptive response in non-sensitized mice, whereas tramadol in doses of 0.5 and 1 μ g/mouse induced analgesia in morphine-sensitized mice. Co-injection of a non-effective dose of tramadol or morphine (0.25 μ g/mouse) with different doses of morphine or tramadol (0.25, 0.5, and 1 μ g/mouse) respectively potentiated the analgesic effect of the previous drug. An isobolographic analysis of data was performed, indicating a synergistic interaction between morphine and tramadol in non-sensitized and morphine-sensitized mice. Our data indicated that both morphine and tramadol elicit more anti-nociceptive response in morphine sensitized mice; there is a synergistic effect between morphine and tramadol upon induction of analgesic effect in non-sensitized and morphine-sensitized mice.

Key words: sensitization, morphine, tramadol, analgesic effect, mice

INTRODUCTION

Pain is one of the outputs of the nociceptive mechanism, which is of major importance to survive (Emam et al., 2016). Opioid drugs are used worldwide for the treatment of moderate-to-severe pain behavior. They induce analgesia through the activation of μ , δ , or κ opioid receptors (Chefer and Shippenberg, 2009; Ochiai et al., 2016; Brodin et al., 2018). Nevertheless, the use

of opioid drugs has been limited due to their negative effects for example abuse liability (Alsalem et al., 2019). Combining various analgesic drugs for increasing drug efficacy is a suggested strategy, proposed to obtain optimal therapeutic influences. These strategies could decrease the drug side effects concomitant with improving their efficacy (Zeraati et al., 2014). In this regard, concurrent application of anti-nociceptive drugs can lead to achieving additive or synergistic effects. Particularly using lower dosages of drugs can

lead to lower the risk of negative side effects (Zeraati et al., 2014; Alsalem et al., 2019). Morphine and tramadol elicits analgesia via various mechanisms of action. Morphine activates μ -opioid receptors without the need for metabolic change to an active metabolite. The non-opioid mechanisms of tramadol induce by inhibition of noradrenaline and serotonin uptake. These different mechanisms produce a potent anti-nociceptive effect in the experimental model and should also provide reliable pain management in the clinical situation (Kogel et al., 2014).

It has been revealed that repeated application of morphine followed by a period of drug-free treatment can produce sensitization and can cause the long-lasting augmentation of morphine behavioral effects (Zarrindast and Rezayof, 2004). The circuitry involved in sensitization is complex since sensitization includes a cascade of events involving various neurotransmitter systems and some brain areas (Kadivar et al., 2014).

Likely approaches to decline the side effects of opioid treatments consist of the use of drugs combination that induces analgesia by diverse mechanisms of action and the use of lowering dose of each drug allows decreasing toxicity (Capuano et al., 2011). This strategy has been used for treating numerous illnesses for example cancer and cardiovascular illnesses, and developing evidence suggest the validity of this strategy for pain treatment (Smith, 2008; Thorn et al., 2011). Based on this respect, this research was designed to study the possible additive or synergistic anti-nociceptive effect of morphine and tramadol by using tail-flick in non-sensitized and morphine-sensitized mice.

Animals

Male albino NMRI mice (obtained from the animal house of University of Tehran, Tehran) weighing 22–30 g were used in the experiments. The mice were kept under controlled laboratory conditions in a room with a 12:12 h light/dark cycle (lights on 7:00 h) and a temperature of $22 \pm 2^\circ\text{C}$. Food and water were accessible ad libitum up to the time of testing. The mice were allowed to habituate to the laboratory conditions for one week previous to surgery. 288 male mice randomly divided in 36 groups and each group included of 8 mice. Mice were handled about 5 min per day for one week previous to behavioral testing. The experiments were carried out between 8:00 a.m. and 1:00 p.m. All procedures for the treatment of mice were confirmed by the Research and Ethics Committee of the University of Tehran and were performed under

the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Drugs

The drugs used in the experiments were morphine sulfate (Temad, Tehran, Iran), and tramadol hydrochloride sulfate (Temad, Tehran, Iran). All compounds were dissolved in physiological saline (0.9%) immediately before their use. Morphine was injected subcutaneously (s.c.) and intracerebroventricularly (i.c.v.) but tramadol was administered only i.c.v. Control mice received saline. For the induction of morphine sensitivity, it was s.c. administered (1 ml/kg). In order to i.c.v. microinjections, drugs were injected into the lateral ventricle using a 2 μl Hamilton micro-syringe (1 μl /mouse). The dose of each drug was selected upon our previous studies (Zarrindast and Rezayof, 2004; Zarrindast et al., 2008; Farahmandfar et al., 2011; Niknamfar et al., 2019).

Surgery and microinjection procedures

For the central microinjection of drugs, the mice were implanted with a 22-gauge stainless steel guide cannula aimed at the lateral ventricle. Implantation was performed under ketamine-xylazine (100 mg/kg ketamine-5 mg/kg xylazine mixture, intraperitoneally) anesthesia, and was done at least 5–7 days before behavioral testing. The coordinates were used 0.9 mm posterior to the bregma, 1.5 mm lateral to the midline, and 2 mm below the top of the skull (Paxinos and Franklin, 2001). The cannula was fixed to the skull using one screw and dental acrylic. A stylet was inserted into the cannula to preserve its patent previous to microinjections. Drug microinjections were carried out by a 27-gauge stainless steel needle (1 mm longer than the guide cannula) attached to a Hamilton micro-syringe through polyethylene tubing. The mice were quietly held by hand; microinjections lasted for 60 s and the cannula was left in place for an extra 60 s to evade the backflow of the solution. Previous to the experiments, the mice had at least 5 days recovery period.

Tail-flick test

A tail-flick apparatus was used for studying the nociceptive reaction to thermal stimulation (M.T9500, Borj Sanat Company, Tehran, Iran). The reaction time between the beginning of the heat stimulus and the

removal of the tail from the heat source was recorded through a sensor as the tail-flick latency. Because the tail-flick latency time (s) normally depends on the proximal-distal location of heating on the tail, the tail was marked with a line in 1 cm increments beginning at the tip, for a total of 5 increments. Each mouse was quietly wrapped in a soft towel and the dorsal surface of the mouse tail from its distal end was instantly placed in the apparatus every 15 min (for 60 min) after the drug/saline infusions. The heat source and a timer were activated simultaneously via a pedal. Both were finished automatically through a tail movement which exposed a photocell below the tail and/or via the experimenter at the end of a 10 s cut-off time. It is important to consider that this cut-off time was set to avoid skin damage. To assess the sensitivity of each mouse to nociceptive stimulus, we measured the animal's tail-flick latency prior to drug injection as a baseline pain threshold. The mice were tested twice in a 15 min interval and the mean of this was calculated as baseline latency. Light intensity was used to find baseline tail-flick latency of 2–4 s. All results were normalized for pre-administration baseline. Individual tail withdrawal latencies were converted to the percentage of maximum possible effect (%MPE) by the following formula: $\%MPE = [(test\ latency - baseline\ latency) / (cut-off\ latency - baseline\ latency)] \times 100$. There were no significant differences in baseline tail-flick latencies between the experimental groups previous to the administration of the drugs and/or saline. For all data, the area under the curve (AUC) of %MPE vs. time was evaluated from 0 to 60 min using the trapezoidal rule to definite the overall magnitude and duration of effect for the tail-flick test.

Experimental design

Experiment 1

In this experiment, the effect of morphine administration on tail-flick latency was measured in non-sensitized and morphine-sensitized mice. Four groups of mice were i.c.v. injected with saline or different doses of morphine (0.25, 0.5, and 1 µg/mouse). The other four groups were sensitized by morphine. To induce sensitization to morphine, the mice received morphine (10, 15, and 20 mg/kg, s.c.) once daily for 3 days (days 1–3) in the colony room, followed by 5 days of no drug treatment (days 4–8). On day 9 (test day), these mice received morphine (5 mg/kg, s.c.) 15 min before i.c.v. microinjection of saline or different doses of morphine (0.25, 0.5, and 1 µg/mouse). The tail-flick latency of each mouse was tested on the tail-flick ap-

paratus. Table 1 explains the protocol and experimental groups.

Experiment 2

This experiment examined the effect of tramadol administration on tail-flick latency in non-sensitized and morphine-sensitized mice. Eight groups of mice were used. Four groups of animals were i.c.v. injected with saline or different doses of tramadol (0.25, 0.5, and 1 µg/mouse). The other four groups were sensitized by morphine as mentioned above. On the test day (day 9), morphine-sensitized mice received morphine (5 mg/kg, s.c.) 15 min before i.c.v. infusion of saline or different doses of tramadol (0.25, 0.5, and 1 µg/mouse).

Experiment 3

In this experiment, the effect of tramadol and morphine co-administration on tail-flick latency was assessed in non-sensitized and morphine-sensitized mice. Four groups of animals were microinjected with saline or different doses of tramadol (0.25, 0.5, and 1 µg/mouse; i.c.v.) plus a single dose of morphine (0.25 µg/mouse; i.c.v.). The other four groups which sensitized by morphine received morphine (5 mg/kg, s.c.) 15 min before i.c.v. co-injection of saline or different doses of tramadol (0.25, 0.5, and 1 µg/mouse) plus a single dose of morphine (0.25 µg/mouse; i.c.v.).

Experiment 4

This experiment examined the effect of morphine and tramadol co-injection on tail-flick latency in non-sensitized and morphine-sensitized mice. Four groups of mice were microinjected with saline or different doses of morphine (0.25, 0.5, and 1 µg/mouse; i.c.v.) plus a single dose of tramadol (0.25 µg/mouse; i.c.v.). The other four groups which sensitized by morphine received morphine (5 mg/kg, s.c.) 15 min before i.c.v. co-administration of saline or different doses of morphine (0.25, 0.5, and 1 µg/mouse) plus a single dose of tramadol (0.25 µg/mouse; i.c.v.).

Experiment 5

To confirm whether morphine and tramadol co-administration would exert additive or synergistic effects on their induced analgesic effect, the isobolographic analysis was performed to compare the theoretical and experimental ED₅₀ of the drugs when injected together. According to the dose-response curve of morphine and tramadol, animals of non-sensitized and morphine-sensitized mice received administration of

Table 1. The table describes protocol and experimental groups.

Day of experiment	Experimental design	Fig.	Drug injection (i.c.v)	Effect on pain
1	Saline (1 ml/kg); non-sensitized Morphine (10 mg/kg, s.c.); morphine-sensitized	-	-	-
2	Saline (1 ml/kg); non-sensitized Morphine (15 mg/kg, s.c.); morphine-sensitized	-	-	-
3	Saline (1 ml/kg); non-sensitized Morphine (20 mg/kg, s.c.); morphine-sensitized	-	-	-
4-8	No drug treatment	-	-	-
9	Saline (1 ml/kg); non-sensitized Morphine (5 mg/kg, s.c.); morphine-sensitized	1	Morphine (0.25, 0.5 and 1 µg/mouse)	Analgesic effect
		2	Tramadol (0.25, 0.5 and 1 µg/mouse)	Analgesic effect
		3	Tramadol (0.25, 0.5 and 1 µg/mouse) + morphine (0.25 µg/mouse)	Analgesic effect
		4	Morphine (0.25, 0.5 and 1 µg/mouse) + tramadol (0.25 µg/mouse)	Analgesic effect
		5	Morphine (0.5 µg/mouse) + tramadol (0.5 µg/mouse) Morphine (0.25 µg/mouse) + tramadol (0.25 µg/mouse) Morphine (0.125 µg/mouse) + tramadol (0.125 µg/mouse)	Synergistic effect on analgesia

morphine 0.5 µg/mouse + tramadol 0.5 µg/mouse, morphine 0.25 µg/mouse + tramadol 0.25 µg/mouse and morphine 0.125 µg/mouse + tramadol 0.125 µg/mouse (Nasehi et al., 2016; 2017).

Histology

The histological results were evaluated on representative sections taken from the mice brain atlas of Paxinos and Franklin (2001). Cannulae were implanted into the left ventricle of a total of 310 mice, however only the data from 288 mice with correct cannulae implants were used in statistical analyses.

Data analysis

The obtained data are indicated as a mean of the percentage of maximal possible effect (% MPE) or areas under the time-course curves (AUC) ± standard error of the mean (S.E.M.). The mean MPE% or AUC in all groups were analyzed by one-way and two-way ANOVA followed by Tukey *post hoc* test to identify differences between the treatments. P value was lower than 0.05 revealed statistically significant.

Moreover, isobolographic analysis was performed to detect the interactions following the injection of the two drugs (Nasehi et al., 2016; 2017). The ED₅₀ of each drug (0.5 µg/mouse for morphine, and 0.5 µg/mouse

for tramadol) was analyzed by linear regression analysis and a combination of the two drugs was injected in a constant dose ratio upon the ED₅₀ values. For drug combinations, the theoretic ED₅₀ is morphine ED₅₀/2 + tramadol ED₅₀/2. Furthermore, experimental values of drug combinations from fixed ratio-calculated were calculated by the regression analysis, after which the experimental ED₅₀ value of the drug combinations was identified (%50 tail-flick latency). The statistical significance of the difference among the theoretical ED₅₀ and experimental ED₅₀ of the drug combinations was recognized by the one-sample t-test. When the experimental ED₅₀ was significantly lower than the theoretical ED₅₀ a synergistic interaction between morphine and tramadol could be concluded, but there was not any difference among them showing additive interaction rather than the synergistic effect (Nasehi et al., 2016; 2017). Differences with P<0.05 among the experimental groups at each point were displayed statistically significant.

RESULTS

The effect of morphine microinjection in non-sensitized and morphine-sensitized mice on tail-flick latency

Fig. 1 indicated the effects of i.c.v. microinjection of morphine (0.25, 0.5, and 1 µg/mouse) in non-sen-

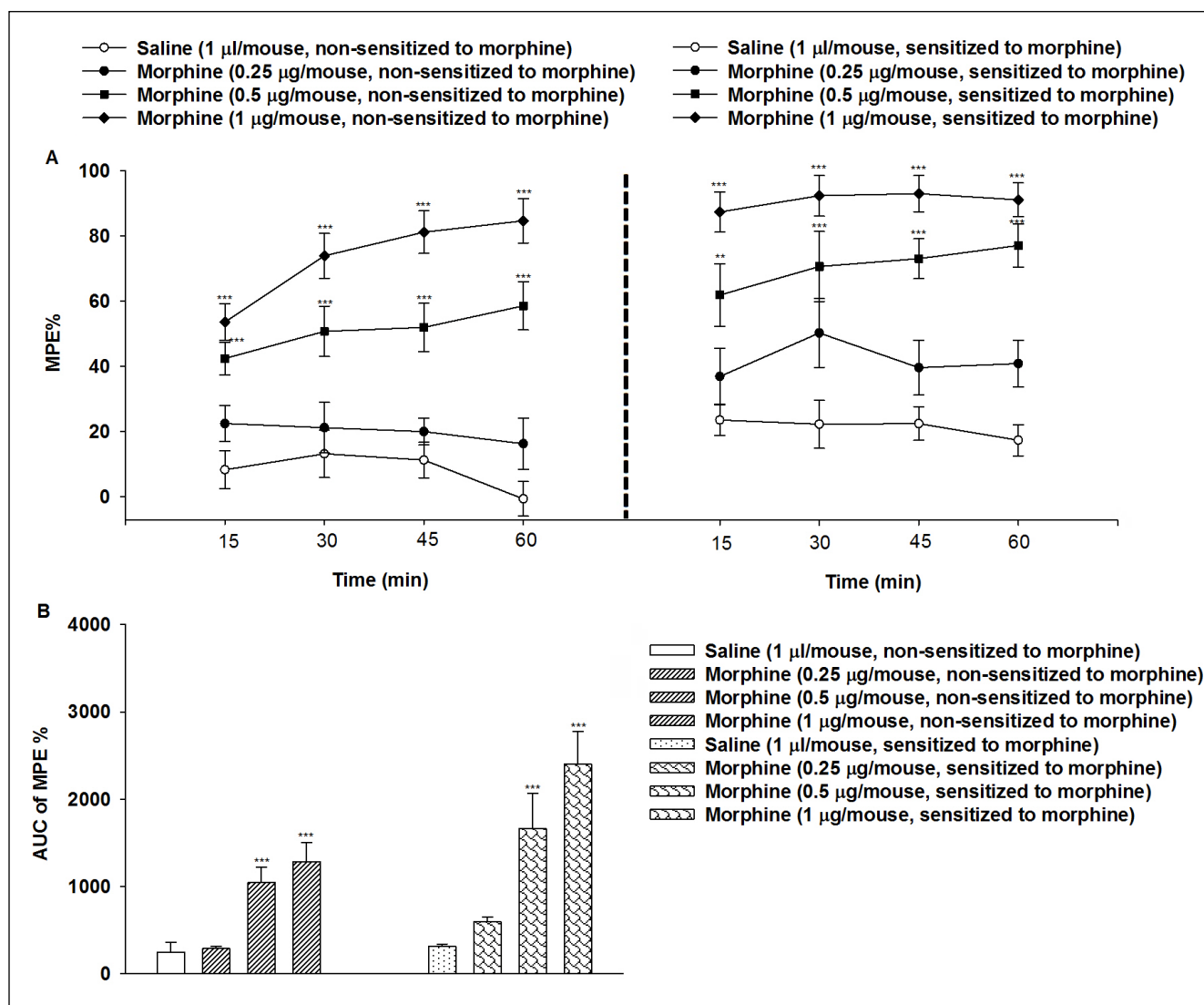


Fig. 1. The effect of morphine i.c.v. administration (0.25, 0.5, and 1 µg/mouse, s.c.) on tail-flick latency in non-sensitized and morphine-sensitized mice. (A; left panel) The percentage of maximal possible effect (MPE%) of morphine in non-sensitized mice at 15, 30, 45, and 60 min after administration. (A; right panel) The percentage of maximal possible effect (MPE%) of morphine in morphine-sensitized mice at 15, 30, 45, and 60 min after injection. Each symbol indicated the mean of MPE% ± S.E.M. n=8, all groups; **P<0.01 and ***P<0.001 as compared with saline control group. (B) The area under the curves (AUCs) calculated for %MPEs in a 60-min period in the tail-flick test (left panel for a non-sensitized and right panel for morphine-sensitized mice). Each symbol showed the mean of AUC ± S.E.M. n=8, all groups; ***P<0.001 as compared with the saline control group.

sitized and morphine-sensitized mice on tail-flick latencies. Two-way ANOVA followed by Tukey's test for repeated measures over time showed that morphine administration increased %MPEs and induced anti-nociceptive response in non-sensitized [time effect: $F_{(3,84)}=24.012$, $P<0.001$; treatment effect: $F_{(3,28)}=5.403$, $P<0.01$ and treatment × time interaction: $F_{(9,84)}=9.818$, $P<0.001$; Fig. 1A (left panel)] and morphine-sensitized [time effect: $F_{(3,84)}=29.75$, $P<0.001$; treatment effect: $F_{(3,28)}=2.065$, $P<0.05$ and treatment × time interaction: $F_{(9,84)}=4.385$, $P<0.01$; Fig. 1A (right panel)] mice.

In addition, as shown in Fig. 1B, one-way ANOVA followed by the Tukey's *post-hoc* test for normalized AUC values revealed that morphine (0.5 and 1 µg/mouse) increased the AUC of MPE% in non-sensitized ($F_{(3,28)}=42.455$, $P<0.001$; left panel) and morphine-sensitized ($F_{(3,28)}=82.203$, $P<0.001$; right panel) mice, indicating an analgesic effect. Also, it was revealed that i.c.v. micro-injection of morphine 0.5 and 1 µg/mouse increased the anti-nociceptive effect of morphine (5 mg/kg; s.c.) in morphine-sensitized mice.

The effect of tramadol infusion in non-sensitized and morphine-sensitized mice on tail-flick latency

The effect of i.c.v. injection of tramadol (0.25, 0.5, and 1 $\mu\text{g}/\text{mouse}$) in non-sensitized and morphine-sensitized mice on tail-flick latency is shown in Fig. 2. Two-way ANOVA for repeated measures over time revealed a significant effects of time [$F_{(3,84)}=45.309$, $P<0.001$], but not for the treatment [$F_{(3,28)}=0.949$, $P>0.05$] and also treatment \times time interaction [$F_{(9,84)}=0.200$, $P>0.05$] in tramadol- vs. saline-treated animals. As shown in Fig. 2A (right panel), two-way ANOVA for

repeated measures over time indicated a significant effects of time [$F_{(3,84)}=97.002$, $P<0.001$], but not for the treatment [$F_{(3,28)}=0.980$, $P>0.05$] and also treatment \times time interaction [$F_{(9,84)}=1.252$, $P>0.05$] in morphine-sensitized mice. Regarding the time intervals effect and tramadol effect, Tukey's multiple comparisons indicated that tramadol (1 $\mu\text{g}/\text{mouse}$) at the time intervals of 15 and 60 min after administration enhanced MPE% of non-sensitized mice and tramadol (0.5 and 1 $\mu\text{g}/\text{mouse}$) at the time interval of 15, 30, 45 and 60 min after injection increased MPE% of morphine-sensitized mice, suggesting an anti-nociceptive effect.

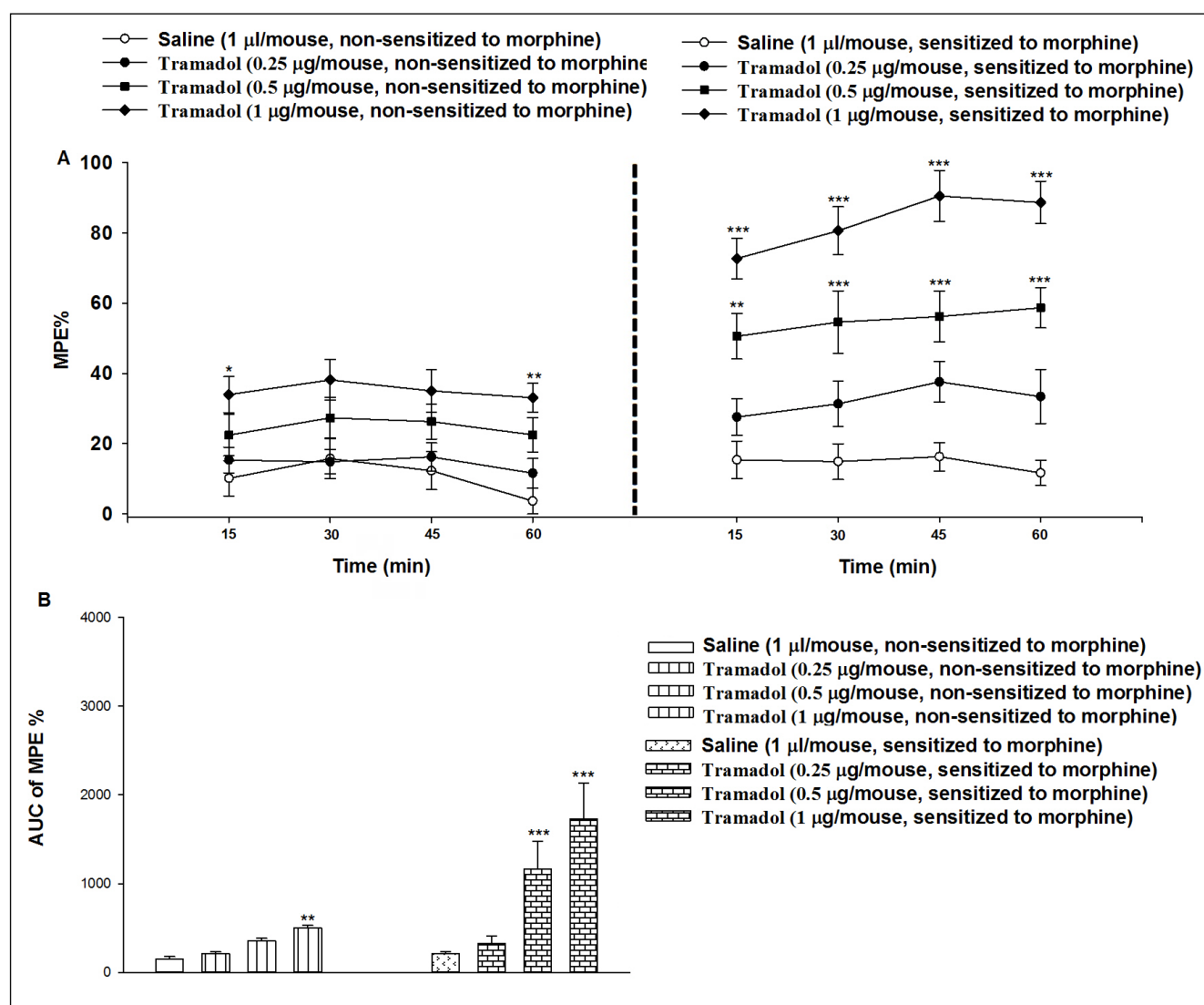


Fig. 2. The effect of tramadol i.c.v. injection (0.25, 0.5, and 1 $\mu\text{g}/\text{mouse}$, s.c.) on tail-flick latency in non-sensitized and morphine-sensitized mice. (A; left panel) The percentage of maximal possible effect (MPE%) of tramadol in non-sensitized mice at 15, 30, 45, and 60 min after infusion. (A; right panel) The percentage of maximal possible effect (MPE%) of tramadol in morphine-sensitized mice at 15, 30, 45, and 60 min after administration. Each symbol displayed the mean of MPE% \pm S.E.M. $n=8$, all groups; * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ as compared with saline control group. (B) The area under the curves (AUCs) calculated for %MPEs in a 60-min period in the tail-flick test (left panel for a non-sensitized and right panel for morphine-sensitized mice). Each symbol presented the mean of AUC \pm S.E.M. $n=8$, all groups; ** $P<0.01$ and *** $P<0.001$ as compared with saline control group.

Furthermore, one-way ANOVA followed by Tukey's *post-hoc* analysis for normalized AUC of MPE% values exhibited that i.c.v. microinjection of the same doses of tramadol, 15 min after saline (1 ml/mouse; i.p.) in non-sensitized mice ($F_{(3,28)}=6.965$, $P<0.01$; Fig. 2B) and morphine (5 mg/kg, s.c.) in morphine-sensitized mice ($F_{(3,28)}=43.386$, $P<0.001$; Fig. 2B) increased AUC of MPE%. It was also found that 0.5 and 1 $\mu\text{g}/\text{mouse}$ of tramadol enhanced the analgesic effect of morphine (5 mg/kg) in morphine-sensitized mice.

The effect of morphine microinjection on tramadol-induced anti-nociceptive effect in non-sensitized and morphine-sensitized mice on tail-flick latency

Fig. 3 showed the effects of i.c.v. microinjection of morphine (0.25 $\mu\text{g}/\text{mouse}$) on tramadol-induced anti-nociceptive response in non-sensitized and morphine-sensitized mice on tail-flick latencies. Two-way ANOVA followed by Tukey's test for repeated mea-

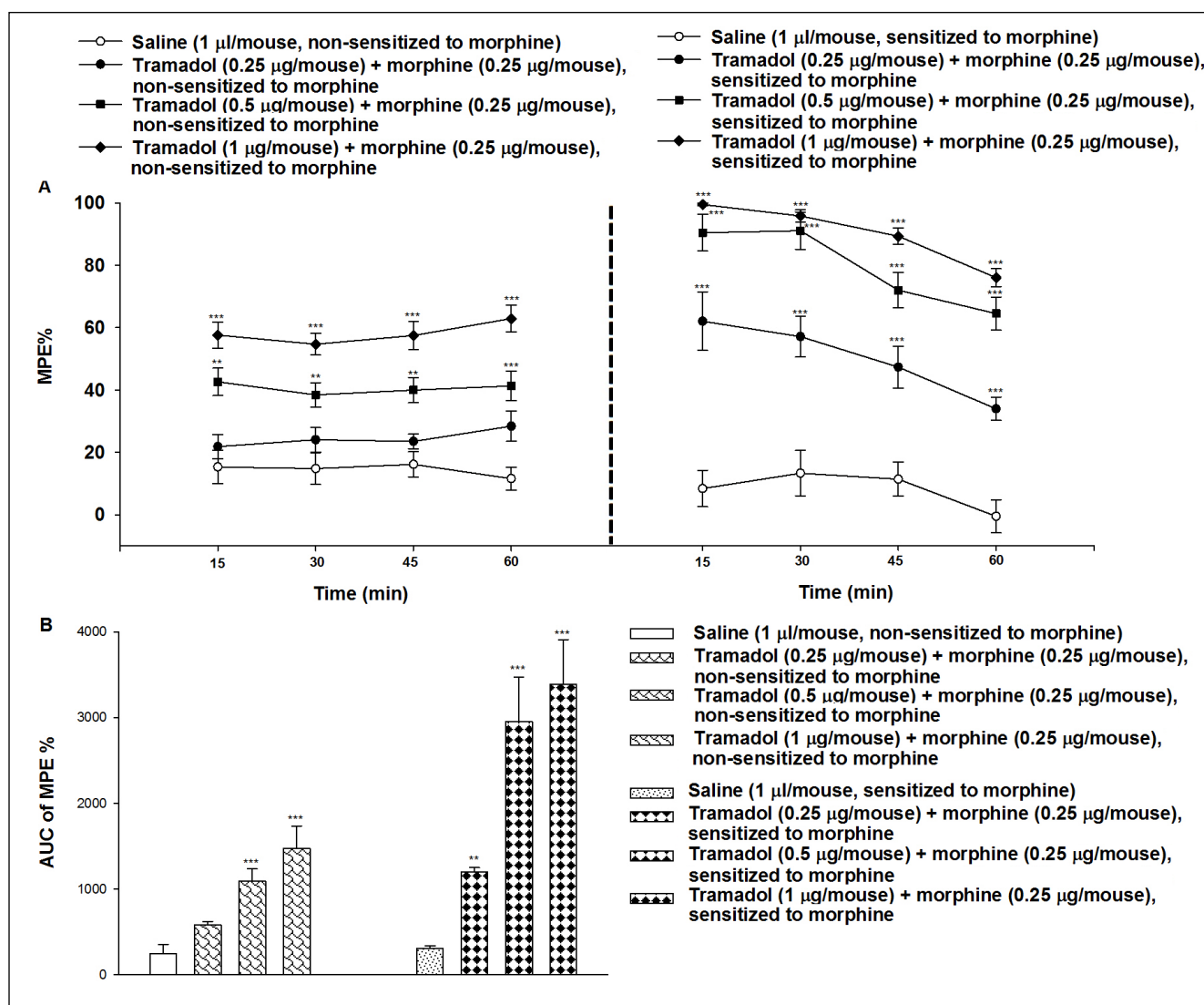


Fig. 3. The effect of i.c.v. co-injection of tramadol and morphine in non-sensitized and morphine-sensitized mice. (A; left panel) showed the percentage of maximal possible effect (MPE%) during a 60-min period after co-injection of tramadol (0.25, 0.5, and 1 $\mu\text{g}/\text{mouse}$) and morphine (0.25 $\mu\text{g}/\text{mouse}$) in non-sensitized mice. (A; right panel) exhibited the percentage of maximal possible effect (MPE%) during a 60-min period afterward co-administration of tramadol (0.25, 0.5, and 1 $\mu\text{g}/\text{mouse}$) and morphine (0.25 $\mu\text{g}/\text{mouse}$) in morphine-sensitized mice. Each symbol represented the mean of MPE% \pm S.E.M. as compared with the control group. $n=8$, all groups; $**P<0.01$ and $***P<0.001$, compared with saline control group. (B) Each value indicated the area under the curve (AUC) of tramadol plus morphine response on tail-flick latency during a 60-min period (left panel for a non-sensitized and right panel for morphine-sensitized mice). $n=8$, all groups; $**P<0.01$ and $***P<0.001$, compared with control group.

tures over time displayed a significant effects of time [$F_{(3,84)}=17.537$, $P<0.001$], but not for the treatment [$F_{(3,28)}=1.409$, $P>0.05$] and also treatment \times time interaction [$F_{(9,84)}=0.452$, $P>0.05$] in drug- vs. saline-treated animals (Fig. 3A; left panel). Additionally, two-way ANOVA for repeated measures over time indicated a significant effects of time [$F_{(3,84)}=75.495$, $P<0.001$], but not for the treatment [$F_{(3,28)}=1.095$, $P>0.05$] and also treatment \times time interaction [$F_{(9,84)}=0.423$, $P>0.05$] in morphine-sensitized mice (Fig. 3A; right panel). According to the time intervals effect and drug effect, Tukey's multiple comparisons indicated that co-ad-

ministration of tramadol (0.5 and 1 $\mu\text{g}/\text{mouse}$) plus morphine (0.25 $\mu\text{g}/\text{mouse}$) at the time intervals of 15, 30, 45 and 60 min after administration enhanced MPE% of non-sensitized mice and morphine-sensitized mice.

In addition, one-way ANOVA followed by Tukey's *post-hoc* analysis for normalized AUC of MPE% values revealed that i.c.v. co-injection of the same doses of tramadol and morphine, 15 min after saline (1 ml/mouse; i.p.) in non-sensitized mice ($F_{(3,28)}=41.351$, $P<0.001$; Fig. 3B) and morphine (5 mg/kg, s.c.) in morphine-sensitized mice ($F_{(3,28)}=70.123$, $P<0.001$; Fig. 3B) enhanced AUC of MPE%.

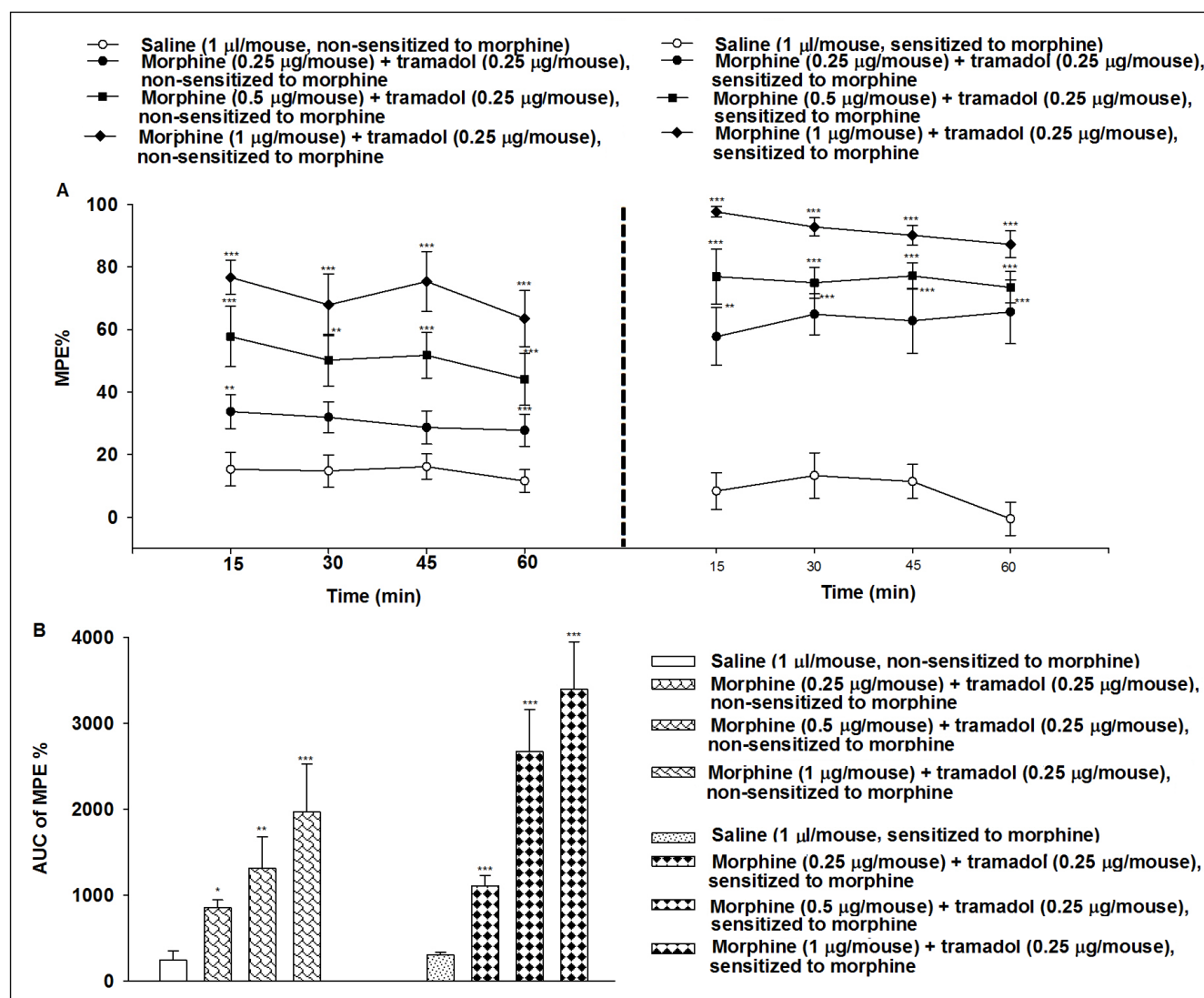


Fig. 4. The effect of i.c.v. co-administration of morphine and tramadol in non-sensitized and morphine-sensitized mice. (A; left panel) displayed the percentage of maximal possible effect (MPE%) during 60-min period after co-administration of morphine (0.25, 0.5, and 1 $\mu\text{g}/\text{mouse}$) and tramadol (0.25 $\mu\text{g}/\text{mouse}$) in non-sensitized mice. (A; right panel) indicated the percentage of maximal possible effect (MPE%) during 60-min period afterward co-injection of morphine (0.25, 0.5, and 1 $\mu\text{g}/\text{mouse}$) and tramadol (0.25 $\mu\text{g}/\text{mouse}$) in morphine-sensitized mice. Each symbol presented the mean of MPE% \pm S.E.M. as compared with control group. $n=8$, all groups; $**P<0.01$ and $***P<0.001$, compared with saline control group. (B) Each value showed the area under the curve (AUC) of morphine plus tramadol response on tail flick latency during 60-min period (left panel for non-sensitized and right panel for morphine-sensitized mice). $n=8$, all groups; $*P<0.05$, $**P<0.01$ and $***P<0.001$, compared with control group.

The effect of tramadol co-infusion on morphine-induced anti-nociceptive effect in non-sensitized and morphine-sensitized mice on tail-flick latency

The effect of i.c.v. co-injection of tramadol (0.25 µg/mouse) on morphine-produced anti-nociceptive response in non-sensitized and morphine-sensitized mice on tail-flick latencies is shown in Fig. 4. Two-way ANOVA followed by Tukey's *post-hoc* test for repeated measures over time exhibited that co-administration of morphine and tramadol increased %MPEs and produced significantly anti-nociceptive in non-sensitized [time effect: $F_{(3,84)}=18.152$, $P<0.001$; treatment effect: $F_{(3,28)}=18.529$, $P<0.001$ and treatment \times time interaction: $F_{(9,84)}=3.479$, $P<0.05$; Fig. 4A (left panel)] and morphine-sensitized [time effect: $F_{(3,84)}=16.845$, $P<0.001$; treatment effect: $F_{(3,28)}=18.937$, $P<0.001$ and treatment \times time interaction: $F_{(9,84)}=5.295$, $P<0.01$; Fig. 4A (right panel)] mice.

Also, one-way ANOVA followed by Tukey's *post-hoc* analysis for normalized AUC of MPE% values showed that i.c.v. co-infusion of the same doses of morphine and tramadol, 15 min after saline (1 ml/mouse; i.p.) in non-sensitized mice ($F_{(3,28)}=58.576$, $P<0.001$; Fig. 4B) and

morphine (5 mg/kg, s.c.) in morphine-sensitized mice ($F_{(3,28)}=84.636$, $P<0.001$; Fig. 4B) increased AUC of MPE%.

The synergistic effect between morphine and tramadol on anti-nociceptive effect in non-sensitized and morphine-sensitized mice

The theoretical additive line showed that at all points, morphine and tramadol combination produced an effect of theoretical %50 tail-flick latency (theoretical ED50) according to an additive interaction (Fig. 5). One sample t-test revealed that there is a significant difference between experimental ED50 and theoretical ED50. Our data proposed a synergistic effect of morphine and tramadol administration upon induction of anti-nociceptive effect in non-sensitized and morphine-sensitized mice (Fig. 5).

DISCUSSION

Opioid drugs, especially agonists of the μ -receptor subtype, are extensively used to treat moderate to severe pain (Al-Hasani and Bruchas, 2011; Pasternak and

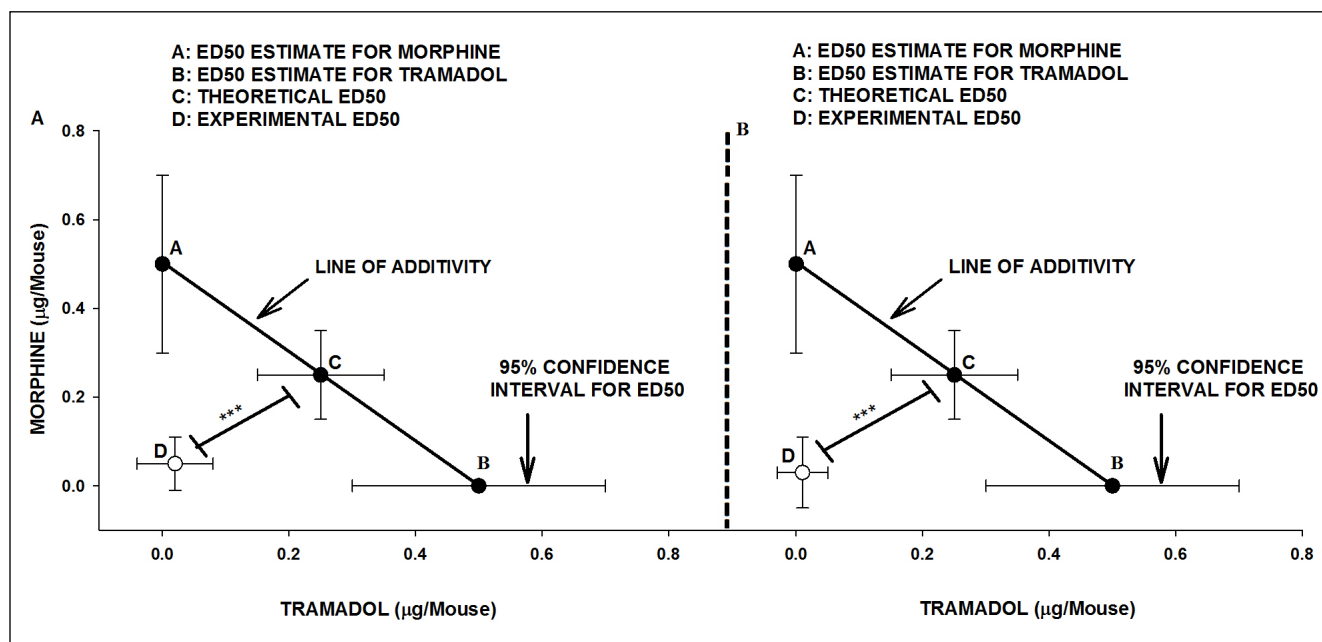


Fig. 5. The isobologram analysis of the effects of drug administration revealed the synergistic effect of i.c.v. injection of morphine and tramadol to induction analgesic effect. The foci A and B mean indicated the ED50 of morphine and tramadol, respectively. In brief, the ED50 of each drug (0.5 µg/mouse for morphine and 0.5 µg/mouse for tramadol) was calculated by linear regression analysis. The oblique line between A and B is the theoretic additive effect line of morphine and tramadol administrations. The focus C, in the middle of the line, is the theoretical ED50 of the drug combination, which is recorded from the individual drug ED50. The focus D, far below the line, is the experimental ED50 of drugs combination, which is obtained after drug administration. Statistical analysis demonstrated that there is a significant difference between experimental ED50 and theoretical ED50 points, suggesting a synergistic effect of the injection of the drug ($n=8$, all groups; left panel for a non-sensitized and right panel for morphine-sensitized mice). ED50, effective dose 50.

Pan, 2011). Opioid receptors are distributed at many sites along the pain-processing pathways, including both the central (spinal cord and several supra-spinal nuclei) and peripheral (dorsal root ganglion and peripheral nerve terminals) nervous system (Bigliardi-Qi et al., 2004; Khalefa et al., 2012). The current study found that i.c.v. administration of morphine prolonged the tail-flick latency in non-sensitized and morphine-sensitized mice, showing the analgesic effect of the drug. Interestingly, the analgesic response of morphine-sensitized mice was more than non-sensitized mice. Because opioid receptors induce analgesic effect (Kosarmadar et al., 2015; Sanchez-Fernandez et al., 2014; Zeng et al., 2013), one may suggest that s.c. and i.c.v. administration of morphine in morphine-sensitized mice induces a powerful anti-nociceptive effect by activating the central and peripheral nervous system (Bigliardi-Qi et al., 2004; Khalefa et al., 2012) or modifying synaptic structures (Robinson and Kolb, 1997; 1999). Furthermore, behavioral sensitization leads to multiple adaptive neuronal responses such as permanent changes in synaptic structures (Robinson and Kolb, 1997; 1999). The adaptive reactions need changed gene expression (Nestler, 2000). A number of these adaptive processes may so serve as acquired molecular twitches, making a subject prone to increase dependence on chemical substances on repeated usage (Vekovischeva et al., 2001). The results of the present investigation are in agreement with the findings of the previous researches which have demonstrated that morphine administration (centrally or peripherally) induced anti-nociceptive response through the stimulation of μ -opioid receptors (Kosarmadar et al., 2015; Sanchez-Fernandez et al., 2014; Zeng et al., 2013). The analgesic effects of morphine may stem from the: acting directly on the presynaptic neuron within the dorsal horn to decrease substance P by suppression of N-type voltage-gated calcium channel activity (Takasusuki and Yaksh, 2011); inhibiting the presynaptic release of glutamate (Schepers et al., 2008); preventing the GABA inhibitory interneurons (Heinricher et al., 1994); decreasing the release of calcitonin gene-related peptide (CGRP) from primary afferents (in mice model) (Baillie et al., 2015); activating potassium channels on second-order neurons, producing hyperpolarization, thereby decreasing action potential transmission at the pre-synaptic level; acting on the nicotinic and muscarinic acetylcholine receptors in the spinal cord, contributing meaningfully to the analgesic effects (Chen and Pan, 2003); acting on the spinal cord via the periaqueductal gray and the rostral ventromedial medulla descending modulation (Heinricher et al., 2009).

Moreover, the results of the present research indicated that i.c.v. microinjection of tramadol en-

hanced the tail-flick latency in non-sensitized and morphine-sensitized mice, displaying the anti-nociceptive effect of the drug. Tramadol is a broadly used analgesic agent (Cha et al., 2014; Grond and Sablotzki, 2004) which its analgesic effects are mediated by a combination of μ -opioid agonist effects and norepinephrine and serotonin reuptake inhibition (Kaneko et al., 2014; Mintzer et al., 2010). Tramadol by activation of the μ -opioid receptor may induce the analgesic effect. However, the affinity of tramadol for the μ -opioid receptor is weak, approximately 6000-fold less than that of morphine (Aarts et al., 2012). Monoamine reuptake inhibition may also participate in the analgesic response of tramadol through inhibiting pain transmission in the CNS (Minami et al., 2015; Zhang et al., 2012). Tramadol used for acute- and chronic pain treatment (Lewis and Han, 1997; Li et al., 2017). Clinical studies have confirmed the efficacy of tramadol in the management of cancer pain (Gonul et al., 2015; Leppert, 2009), and neuropathic pain (Christoph et al., 2007; Hollingshead et al., 2006). In basic researches in animals, the anti-nociceptive effects of tramadol on heat pain (Raffa et al., 1992), chemical pain (Oliva et al., 2002), visceral pain (Oyama et al., 2012), and neuropathic hyperalgesia (Tsai et al., 2000) have been widely studied. Because of tramadol lower susceptibility of addiction than morphine, it is usually used for patients in the postsurgical period and also in a patient with chronic pain syndromes (Hosseini-Sharifabad et al., 2016). It has been demonstrated that tramadol shows dose-dependent and time-dependent anti-nociceptive effects on acute thermal pain in mice using the models of nociception most applied, the hot plate, and tail-flick test (Aydin et al., 2012). A possible clarification for this might be that the reaction of animals depends on the various behavioral tests, route of application, and doses of the drugs.

In the next section of our study, we found that co-administration of a non-effective dose of tramadol or morphine with diverse doses of morphine or tramadol respectively potentiated the anti-nociceptive effect of the previous drug. Interestingly, our data revealed a synergistic effect of morphine and tramadol upon induction of analgesic effect, by isobolographic analysis. As mentioned previously, morphine and tramadol act directly on the μ -opioid receptors without the need for metabolic activation and cause a potent anti-nociceptive response in the experimental animals and could also prepare reliable pain management in the clinical studies (Kogel et al., 2014). However, the side effects of opioid treatments are commonly cognitive impairment, tolerance, and dependence (Gepetti and Benemei, 2009) which are typically time- and dose-dependent (Capuano et al., 2011). A possible

strategy to weaken the side effects of opioid treatments includes the use of drugs combination which causing analgesia *via* different mechanisms of action that produces the classical synergistic effect. Furthermore, the use of lowering doses of each drug allows declining overall toxicity (Capuano et al., 2011). This scientifically valid strategy has been successfully used for treating several diseases such as cancer and cardiovascular disorders, and emerging reports propose the validity of this strategy for treating pain (Smith, 2008; Thorn et al., 2011). For instance, the combination of a μ -opioid agonist with another non- μ -opioid analgesic may have enhanced analgesic effectiveness and/or a better safety profile (Smith, 2008). Supplementary support comes from the results that analgesic drugs with dual mechanisms of action (μ -opioid receptor agonist and a second mechanism) tend to have enhanced therapeutic profiles. For example, tramadol is a μ -opioid receptor agonist that also increases serotonin and norepinephrine transmission (Reeves and Burke, 2008). Tramadol is effective in several painful conditions and has relatively low abuse liability, probably due to this unique pharmacological profile (Epstein et al., 2006; Thorn et al., 2011). Moreover, we suggest that a synergistic effect between morphine and tramadol may be involved in the modulation of pain behavior in non-sensitized and morphine-sensitized mice. Nonetheless, more investigations are needed to explain the exact mechanisms of morphine and tramadol in the modulation of pain response in non-sensitized and morphine-sensitized male mice.

REFERENCES

- Aarts E, Helmich RC, Janssen MJ, Oyen WJ, Bloem BR, Cools R (2012) Aberrant reward processing in Parkinson's disease is associated with dopamine cell loss. *Neuroimage* 59: 3339–3346.
- Al-Hasani R, Bruchas MR (2011) Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology* 115: 1363–1381.
- Alsalem M, Altarifi A, Haddad M, Aldossary SA, Kalbouneh H, Aldaoud N, El-Salem K (2019) Antinociceptive and abuse potential effects of cannabinoid/opioid combinations in a chronic pain model in rats. *Brain Sci* 9: 328.
- Aydin ON, Ek RO, Temocin S, Ugur B, Alacam B, Sen S (2012) The antinociceptive effects of systemic administration of tramadol, gabapentin and their combination on mice model of acute pain. *Agri* 24: 49–55.
- Baillie LD, Schmidhammer H, Mulligan SJ (2015) Peripheral μ -opioid receptor mediated inhibition of calcium signaling and action potential-evoked calcium fluorescent transients in primary afferent CGRP nociceptive terminals. *Neuropharmacology* 93: 267–273.
- Bigliardi-Qi M, Sumanovski LT, Buchner S, Rufli T, Bigliardi PL (2004) μ -opiate receptor and Beta-endorphin expression in nerve endings and keratinocytes in human skin. *Dermatology* 209: 183–189.
- Brolin E, Zellerroth S, Jonsson A, Hallberg M, Gronbladh A, Nyberg F (2018) Chronic administration of morphine using mini-osmotic pumps affects spatial memory in the male rat. *Pharmacol Biochem Behav* 167: 1–8.
- Capuano A, De Corato A, Treglia M, Tringali G, Navarra P (2011) Flupirtine antinociception in the rat orofacial formalin test: an analysis of combination therapies with morphine and tramadol. *Pharmacol Biochem Behav* 97: 544–550.
- Cha, HJ, Song MJ, Lee KW, Kim EJ, Kim YH, Lee Y, Jeong HS (2014) Dependence potential of tramadol: behavioral pharmacology in rodents. *Biomol Ther* 22: 558–562.
- Chefer VI, Shippenberg TS (2009) Augmentation of morphine-induced sensitization but reduction in morphine tolerance and reward in delta-opioid receptor knockout mice. *Neuropsychopharmacology* 34: 887–898.
- Chen SR, Pan HL (2003) Antinociceptive effect of morphine, but not μ opioid receptor number, is attenuated in the spinal cord of diabetic rats. *Anesthesiology* 99: 1409–1414.
- Christoph T, Kogel B, Strassburger W, Schug SA (2007) Tramadol has a better potency ratio relative to morphine in neuropathic than in nociceptive pain models. *Drugs R D* 8: 51–57.
- Emam AH, Hajesfandiari N, Shahidi S, Komaki A, Ganji M, Sarihi A (2016) Modulation of nociception by medial pre-optic area orexin receptors and its relation with morphine in male rats. *Brain Res Bull* 127: 141–147.
- Epstein DH, Preston KL, Jasinski DR (2006) Abuse liability, behavioral pharmacology, and physical-dependence potential of opioids in humans and laboratory animals: lessons from tramadol. *Biol Psychol* 73: 90–99.
- Farahmandfar M, Zarrindast MR, Kadivar M, Karimian SM, Naghdi N (2011) The effect of morphine sensitization on extracellular concentrations of GABA in dorsal hippocampus of male rats. *Eur J Pharmacol* 669: 66–70.
- Geppetti P, Benemei S (2009) Pain treatment with opioids: achieving the minimal effective and the minimal interacting dose. *Clin Drug Investig* 29: 3–16.
- Gonul O, Satilmis T, Ciftci A, Sipahi A, Garip H, Goker K (2015) Comparison of the effects of topical ketamine and tramadol on postoperative pain after mandibular molar extraction. *J Oral Maxillofac Surg* 73: 2103–2107.
- Grond S, Sablotzki A (2004) Clinical pharmacology of tramadol. *Clin Pharmacokinet* 43: 879–923.
- Heinricher MM, Morgan MM, Tortorici V, Fields HL (1994) Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. *Neuroscience* 63: 279–288.
- Heinricher MM, Tavares I, Leith JL, Lumb BM (2009) Descending control of nociception: Specificity, recruitment and plasticity. *Brain Res Rev* 60: 214–225.
- Hollingshead J, Duhmke RM, Cornblath DR (2006) Tramadol for neuropathic pain. *Cochrane Database Syst Rev*: CD003726.
- Hosseini-Sharifabad A, Rabbani M, Sharifzadeh M, Bagheri N (2016) Acute and chronic tramadol administration impair spatial memory in rat. *Res Pharm Sci* 11: 49–57.
- Kadivar M, Farahmandfar M, Ranjbar FE, Zarrindast MR (2014) Increased calcium/calmodulin-dependent protein kinase II activity by morphine-sensitization in rat hippocampus. *Behav Brain Res* 267: 74–82.
- Kaneko K, Umehara M, Homan T, Okamoto K, Oka M, Oyama T (2014) The analgesic effect of tramadol in animal models of neuropathic pain and fibromyalgia. *Neurosci Lett* 562: 28–33.
- Khalefa BI, Shaqura M, Al-Khrasani M, Furst S, Mousa SA, Schafer M (2012) Relative contributions of peripheral versus supraspinal or spinal opioid receptors to the antinociception of systemic opioids. *Eur J Pain* 16: 690–705.
- Kogel B, Terlinden R, Schneider J (2014) Characterisation of tramadol, morphine and tapentadol in an acute pain model in Beagle dogs. *Vet Anaesth Analg* 41: 297–304.
- Kosarmadar N, Ghasemzadeh Z, Rezayof A (2015) Inhibition of microglia in the basolateral amygdala enhanced morphine-induced antinociception: Possible role of GABAA receptors. *Eur J Pharmacol* 765: 157–163.
- Leppert W (2009) Tramadol as an analgesic for mild to moderate cancer pain. *Pharmacol Rep* 61: 978–992.
- Lewis KS, Han NH (1997) Tramadol: a new centrally acting analgesic. *Am J Health Syst Pharm* 54: 643–652.

- Li XQ, Ye ZM, Wang JB, Fan CR, Pan AW, Li C, Zhang RB (2017) Mucoadhesive buccal films of tramadol for effective pain management. *Rev Bras Anesthesiol* 67: 231–237.
- Minami K, Ogata J, Uezono Y (2015) What is the main mechanism of tramadol? *Naunyn Schmiedeberg Arch Pharmacol*, 388: 999–1007.
- Mintzer MZ, Lanier RK, Lofwall MR, Bigelow GE, Strain EC (2010) Effects of repeated tramadol and morphine administration on psychomotor and cognitive performance in opioid-dependent volunteers. *Drug Alcohol Depend* 111: 265–268.
- Nasehi M, Morteza-Zadeh P, Khakpai F, Zarrindast MR (2016) Additive effect of harmaline and muscimol for memory consolidation impairment in inhibitory avoidance task. *Neuroscience* 339: 287–295.
- Nasehi M, Ostadi E, Khakpai F, Ebrahimi-Ghiri M, Zarrindast MR (2017) Synergistic effect between D-AP5 and muscimol in the nucleus accumbens shell on memory consolidation deficit in adult male Wistar rats: an isobologram analysis. *Neurobiol Learn Mem* 141: 134–142.
- Nestler EJ (2000) Genes and addiction. *Nat Genet* 26: 277–281.
- Niknamfar S, Nouri Zadeh-Tehrani S, Sadat-Shirazi MS, Akbarabadi A, Rahimi-Movaghar A, Zarrindast MR (2019) μ -opioid receptor in the CA1 involves in tramadol and morphine cross state-dependent memory. *Neurosci Lett* 705: 177–182.
- Ochiai W, Kaneta M, Nagae M, Yuzuhara A, Li X, Suzuki H, Sugiyama K (2016) Mice with neuropathic pain exhibit morphine tolerance due to a decrease in the morphine concentration in the brain. *Eur J Pharm Sci* 92: 298–304.
- Oliva P, Aurilio C, Massimo F, Grella A, Maione S, Grella E, Berrino L (2002) The antinociceptive effect of tramadol in the formalin test is mediated by the serotonergic component. *Eur J Pharmacol* 445: 179–185.
- Oyama T, Homan T, Kyotani J, Oka M (2012) Effect of tramadol on pain-related behaviors and bladder overactivity in rodent cystitis models. *Eur J Pharmacol* 676: 75–80.
- Pasternak G, Pan YX (2011) μ opioid receptors in pain management. *Acta Anaesthesiol Taiwan* 49: 21–25.
- Paxinos G, Franklin KBJ (2001) *The Mouse Brain in Stereotaxic Coordinates*. 2nd Ed Academic Press, San Diego.
- Raffa RB, Friderichs E, Reimann W, Shank RP, Codd EE, Vaught JL (1992) Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an 'atypical' opioid analgesic. *J Pharmacol Exp Ther* 260: 275–285.
- Reeves RR, Burke RS (2008) Tramadol: basic pharmacology and emerging concepts. *Drugs Today* 44: 827–836.
- Robinson TE, Kolb B (1997) Persistent structural modifications in nucleus accumbens and prefrontal cortex neurons produced by previous experience with amphetamine. *J Neurosci* 17: 8491–8497.
- Robinson TE, Kolb B (1999) Morphine alters the structure of neurons in the nucleus accumbens and neocortex of rats. *Synapse* 33: 160–162.
- Sanchez-Fernandez C, Montilla-Garcia A, Gonzalez-Cano R, Nieto FR, Romero L, Artacho-Cordon A, Cobos EJ (2014) Modulation of peripheral μ -opioid analgesia by sigma1 receptors. *J Pharmacol Exp Ther* 348: 32–45.
- Schepers RJ, Mahoney JL, Shippenberg TS (2008) Inflammation-induced changes in rostral ventromedial medulla μ and kappa opioid receptor mediated antinociception. *Pain* 136: 320–330.
- Smith HS (2008) Combination opioid analgesics. *Pain Physician* 11: 201–214.
- Takasusuki T, Yaksh TL (2011) Regulation of spinal substance p release by intrathecal calcium channel blockade. *Anesthesiology* 115: 153–164.
- Thorn DA, Zhang Y, Peng BW, Winter JC, Li JX (2011) Effects of imidazoline I(2) receptor ligands on morphine- and tramadol-induced antinociception in rats. *Eur J Pharmacol* 670: 435–440.
- Tsai YC, Sung YH, Chang PJ, Kang FC, Chu KS (2000) Tramadol relieves thermal hyperalgesia in rats with chronic constriction injury of the sciatic nerve. *Fundam Clin Pharmacol* 14: 335–340.
- Vekovischeva OY, Zamanillo D, Echenko O, Seppala T, Uusi-Oukari M, Honkanen A, Korpi ER (2001) Morphine-induced dependence and sensitization are altered in mice deficient in AMPA-type glutamate receptor-A subunits. *J Neurosci* 21: 4451–4459.
- Zarrindast MR, Hoghooghi V, Rezayof A (2008) Inhibition of morphine-induced amnesia in morphine-sensitized mice: involvement of dorsal hippocampal GABAergic receptors. *Neuropharmacology* 54: 569–576.
- Zarrindast MR, Rezayof A (2004) Morphine state-dependent learning: sensitization and interactions with dopamine receptors. *Eur J Pharmacol* 497: 197–204.
- Zeng P, Chen J X, Yang B, Zhi X, Guo FX, Sun ML, Wei J (2013) Attenuation of systemic morphine-induced analgesia by central administration of ghrelin and related peptides in mice. *Peptides* 50: 42–49.
- Zeraati F, Araghchian M, Farjoo MH (2014) Ascorbic acid interaction with analgesic effect of morphine and tramadol in mice. *Anesth Pain Med* 4: e19529.
- Zhang M, Jing L, Liu Q, Wen RT, Li JX, Li YL, Liang JH (2012) Tramadol induces conditioned place preference in rats: interactions with morphine and buprenorphine. *Neurosci Lett* 520: 87–91.