Acta Physiologica Sinica, December 25, 2005, 57 (6): 696-704
http://www.actaps.com.cn

Research Paper

Tramadol and dihydroetorphine produce synergistic analgesic effect and postpones acute opiate tolerance in rats

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Abstract: The present study investigated whether a co-application of tramadol (TRA) and dihydroetorphine (DHE) would exert a synergy in analgesic effect and delay acute tolerance development. Intraperitoneal injection of TRA (in mg) and subcutaneous injection of DHE (in ng) were delivered in fixed proportions (1:6.25, 1:12.5, 1:25, 1:50, 1:100, and 1:200). The effect of analgesia was accessed by tail-flick test and analyzed with isobolographic analysis. For test of acute tolerance, six successive injections of either TRA (20 mg/kg) alone, DHE (1 000 ng/kg) alone, or a combination of TRA (20 mg/kg) and DHE (250 ng/kg) were administered. We found that (1) except for 1 mg: 6.25 ng and 1 mg: 50 ng, combinations, all the other ratios produced a significant synergy in their analgesic effect; (2) the effect of analgesia induced by repeated TRA plus DHE injections lasted significantly longer, indicating a slower onset of acute tolerance. These results indicate that TRA and DHE injections in certain dose ratios can induce synergistic analgesia, which is resistant against the development of acute tolerance.

Key words: tramadol; dihydroetorphine; synergistic effect; analgesia; acute tolerance

曲马朵与二氢埃托啡协同镇痛并推迟大鼠急性耐受

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摘 要： 本文旨在研究曲马朵(tramadol, TRA)和二氢埃托啡(dihydroetorphine, DHE)联合用药是否可产生协同镇痛并延缓耐受的发生。TRA (mg，腹腔注射)与 DHE (ng，皮下注射)按固定比率给药(1∶6.25, 1∶12.5, 1∶25, 1∶50, 1∶100, 1∶200)，用热辐射甩尾法评价镇痛效应，采用等高线法评估药物的协同作用。在急性耐受实验中，连续 6 次注射 TRA (20 mg/kg)、DHE (1 000 ng/kg)或两药的组合(TRA 20 mg/kg + DHE 250 ng/kg)。结果显示：(1) 除 1 mg∶6.25 ng 和 1 mg∶50 ng 两个比例外，其他所有比例用药均产生显著的协同镇痛效应；(2) TRA 与 DHE 联合用药的疗效在连续给药中持续时间明显延长，提示二者联合使用可延缓耐受。以上结果提示：TRA 与 DHE 在一定剂量比范围内可产生协同镇痛效应，并可推迟耐受的形成。

关键词：曲马朵；二氢埃托啡；协同作用；镇痛；急性耐受
中图分类号：Q432; R339.11

Analgesic agents of pure opioid group generally possess high analgesic efficacy[1,2], while they are endowed with undesirable properties, i.e., the unwanted side effects increase with dose[3,4]. Therefore, the combinations of different types of analgesics would be considered a better choice if they could create synergistic analgesia with lower

Received 2005-07-29 Accepted 2005-10-09

This work was supported by the National Natural Science Foundation of China (No.39700188, 30170307, and 30370461), the National Basic Research Priorities Programme of China (2001, No. 50 and 2003CB515407), a grant from the 211 project, and a startup grant for young scientists from the 985 project of Peking University.

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doses and hence less side effects. These include combination of opioids with non-opioids, or typical opioids (e.g., dihydroetorphine) with atypical opioids (e.g., tramadol)[5,6].

Dihydroetorphine (DHE) is one of the strongest analgesic opioid alkaloids known, which is 1 000 to 12 000 times more potent than morphine[7,8]. DHE is primarily a selective opioid μ-receptor agonist also with some κ- and δ-partial agonist effects[9,10]. This has brought to its applications in both veterinary and human medicine[8,11]. However, the duration of its action is short and the analgesic action disappears within 120 min after administration[8]. Therefore, repeated delivering of the drug is necessary to maintain sufficient analgesia, which brings about the problem of acute tolerance[8]. If a combination of DHE in lower doses with another drug could generate satisfactory analgesic effect, it might be able to put off the development of acute tolerance and prolong the duration of analgesic action.

Tramadol (TRA) is a weak opioid agonist with antinociceptive effects through its action on μ-receptor and the inhibition of the neuronal re-uptake of both noradrenaline and serotonin[12,13]. TRA is commonly used in the treatment of severe post-operative pain and as an alternative to opiates due to its low physical dependence[14]. However, TRA also displays certain adverse effect[14], and it also has the tendency to develop tolerance like other opioids. Thus, a reduction of its dose by co-application with other analgesics will be laudable, if they can work synergistically and postpone the development of tolerance.

Several reports have demonstrated that agonists of different opioid receptors can produce analgesic synergy[15,16]. However, it has not been reported whether the combination of DHE and TRA will produce synergistic effect of analgesia, and if so, whether this mixture will be less likely to induce acute tolerance and hence work longer. In the present study, intraperitoneal (i.p.) TRA was combined with subcutaneous (s.c.) DHE to address the aforementioned questions. To analyze the interaction of these two drugs, an isobolographic method was used as the basis of our experimental design and the tool of statistical analysis [17-20].

1 MATERIALS AND METHODS

1.1 *Animals*

Experiments were carried out on 358 adult male and female Wistar rats (weighing 150~300 g) provided by the Animal Centre of Peking University Health Science Centre. The animals were housed six to eight per cage with free access to food (chow pellets) and tap water. Rats were maintained on a natural 12 h light-12 h dark cycle. All experiments have adhered to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). Approval from the Institutional Animal Care and Use Committee at Peking University Health Science Centre was also obtained to perform the described experiments. All efforts had been made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available. Animals were randomly assigned to experimental groups and all observations and treatments were double-blind.

1.2 *Drugs*

Tramadol hydrochloride was provided by Beijing Four-Ring Pharmaceutical Co., Ltd. Dihydroetorphine was a gift from Professor QIN Bo-Yi at the Academy of Military Medical Science of China.

1.3 *Nociceptive test*

All experiments were performed under a room temperature of (20±1) °C. Rats were restrained in a plastic holder with the tail exposed and hanged freely. The nociceptive threshold was measured by the latency of the tail flick responses elicited by radiant heat applied to the lower 1/3 of the tail. The mean tail flick latency (TFL) of three measurements was recorded at 5 min intervals at the start of the experiment, and was taken as the basal threshold. The intensity of heat stimuli in the tail flick test was adjusted so that the animals flicked their tail within 4~6 s. The TFLs taken at 20 min intervals after drug administration were recorded, and the percentage changes from basal thresholds were calculated as the expression of anti-nociceptive effect of these drugs. A cut off limit of +150% above baseline was predetermined in order to avoid unnecessary skin damage.

1.4 *Experimental design and statistical analysis*

Isobolographic analysis for drug-drug interaction was conducted according to the procedure of Tallarida[20]. To perform the isobolographic analysis, TRA and DHE of various doses were administered in fixed ratios (1:6.25, 1:12.5, 1:25, 1:50, 1:100, and 1:200, numbers were in mg : ng), thereby producing a series of dose-response curves. The experimental ED_{50} values (denoted Z*mix) and their corresponding 95% confidence intervals (CI95s) for these drug combinations were calculated. They were then compared with that of the theoretically additive ED_{50} of the combination (Z*add). The calculation of Z*add and the corresponding CI95s has been described in detail by Tallarida [20]. If the CI95s of Z*add and Z*mix do not overlap, then we consider that the effect of the mixture depart from simple additivity. If Z*mix<Z*add, the mixture is synergistic un-

der this ratio; whereas the relation Z*mix > Z*add means sub-additivity.

For acute tolerance, six successive injections of either TRA (20 mg/kg, i.p.), DHE (1 000 ng/kg, s.c.), or a combination of TRA (20 mg/kg, i.p.) and DHE (250 ng/kg, s. c.) were delivered with 80-minute inter-injection intervals. The mean areas under the time-effect curves (MAUC, estimated with the mean effect during the time period) 20~60 min after each injection were calculated. Two-way ANOVA were applied to compare the analgesic effects of different drugs.

2 RESULTS

2.1 ED_{50} for TRA and DHE

One hundred and fifty-two rats were randomly assigned into 13 groups, with 11~12 rats in each group. Male and female rats were approximately evenly used in each group. Six of these groups received i.p. injection of TRA (3.125, 6.25, 12.5, 25, 40, and 50 mg/kg, respectively). The other seven groups received s.c. injection of DHE (187.5, 375, 625, 750, 1 000, 1 500, and 3 000 ng/kg, respectively). The time-effect curves of different TRA or DHE doses were shown in Fig. 1*A* and *B*. The changes of TFL usually approached the baseline 120 min after the administration of drugs except for the two highest doses. Since the maximum effect does not always happen at the same time, and the shapes of the time-effect curves vary with doses, we used the mean area under the time-effect curve (MAUC) within 20~120 min after drug injection as the index of their mean analgesic effect. The dose-response curves for these two drugs were shown in Fig. 1*C* and *D*. The ED_{50}s for TRA and DHE and their corresponding CI95s were obtained from the dose-response curves, which are 23.40 (20.31~26.97) mg/kg and 1 355 (1 107~1 673) ng/kg, respectively.

We also calculated the dose-response curves separately for male and female rats (not shown). The ED_{50}s and CI95s of TRA in male and female rats were 24.78 (19.61~31.31) and 22.21 (18.70~26.38) mg/kg, respectively. The cor-

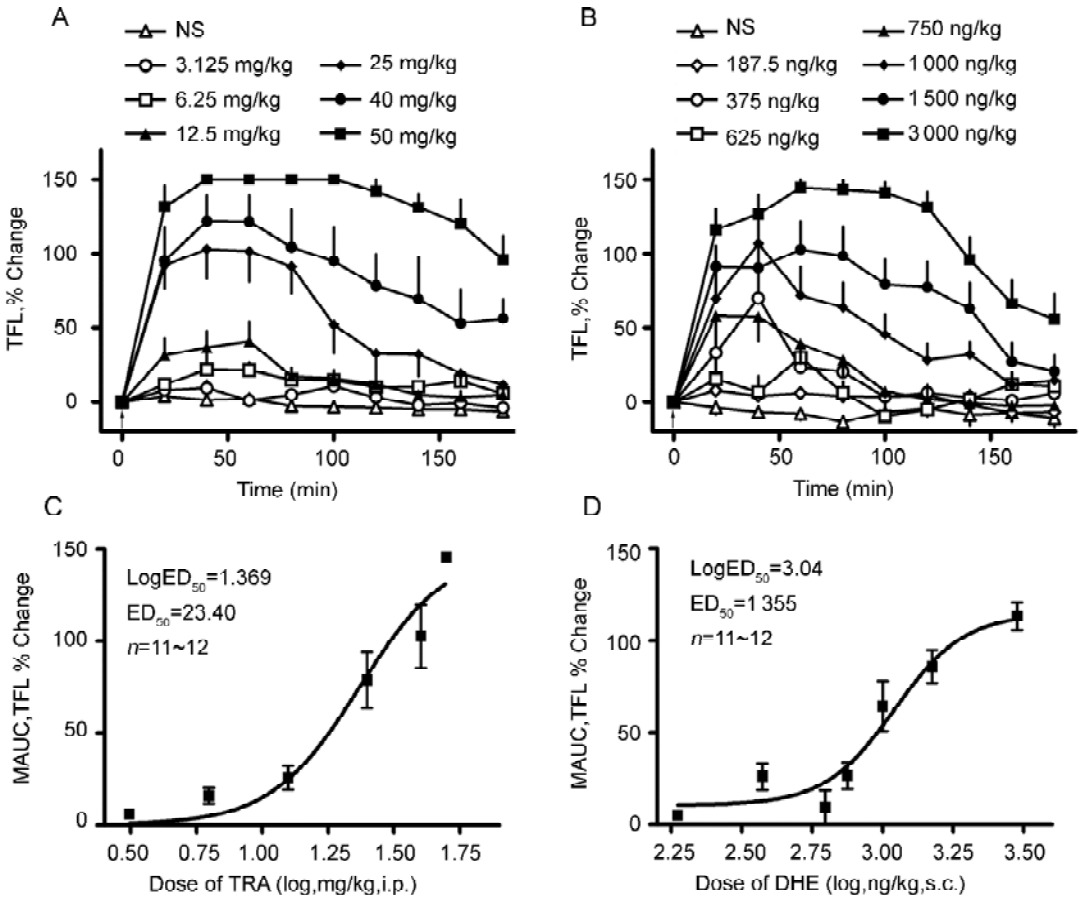

Fig. 1. Time-effect curves of TRA (*A*) and DHE (*B*). Arrows indicate the time of injection (time=0). The dose-response curves are shown in *C* for TRA and *D* for DHE. TRA, tramadol; DHE, dihydroetorphine; TFL, tail-flick latency; NS, normal saline; ED_{50}, 50% effective dose; MAUC, mean area under the curve.

responding values of DHE in male and female rats were 1 575 (1 275~1 947) and 1 140 (944.5~1 376) ng/kg, respectively. None of these values showed any significant gender difference. Thus, we pooled our data from both genders in later analysis throughout the current study.

2.2 Analgesic effect of the TRA-DHE combination

A total of 180 rats were randomly assigned to 27 groups, with 6~8 rats in each group, sex balanced. Rats in each of the groups were given a combination of i.p. TRA/s.c. DHE with the dose ratio fixed to either 1 mg/6.25 ng (5, 10, 20, 40, and 80 mg/kg for TRA in the mixture), 1 mg/12.5 ng (2.5, 5, 10, 20 mg/kg for TRA in the mixture), 1 mg/25 ng (2.5, 5, 10, and 20 mg/kg for TRA in the mixture), 1 mg/ 50 ng (1.25, 2.5, 5, 10, 20, and 40 mg/kg for TRA in the mixture), 1 mg/100 ng (2.5, 5, 10, and 20 mg/kg for TRA in the mixture), or 1 mg/200 ng (2.5, 5, 10, and 20 mg/kg for TRA in the mixture). MAUCs of the time-effect curves of each group were computed as the response index. The dose-response curves of every dose ratio were shown in Fig.2.

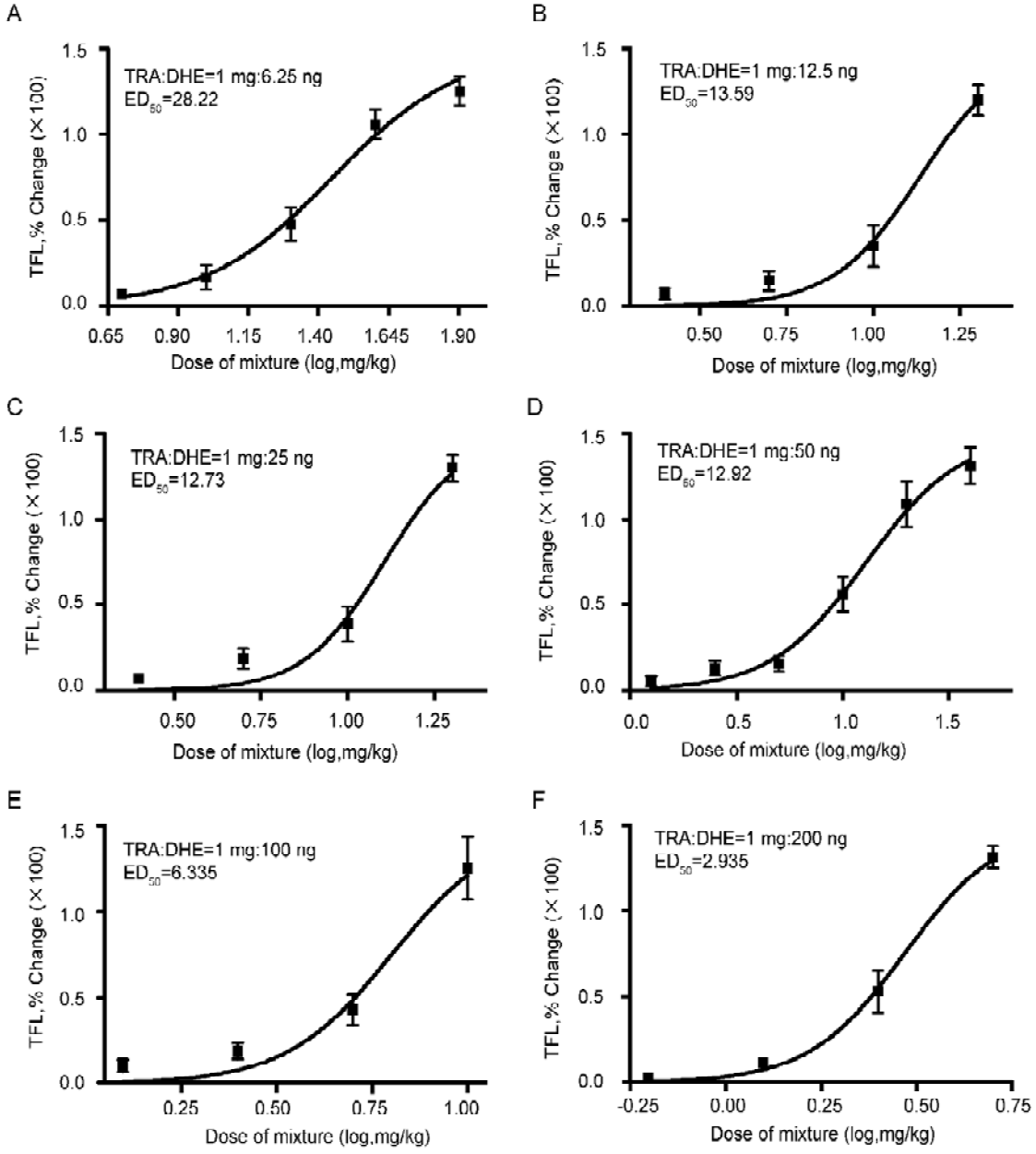

Fig. 2. Dose-response curves of TRA and DHE in combination under 6 different proportions. The ratios of TRA and DHE were 1 mg / 6.25 ng (*A*), 1 mg / 12.5 ng (*B*) , 1 mg / 25 ng (*C*), 1 mg / 50 ng (*D*) , 1 mg / 100 ng (*E*), and 1 mg / 200 ng (*F*). Doses of the combinations were selected in a way that maximal analgesia could be reached with the highest doses. Error bars show the standard error of means. n= 6 ~ 8 in each group. TRA, tramadol; DHE, dihydroetorphine; TFL, tail-flick latency; ED_{50}, 50% effective dose.

The linear isobole of additivity assumes that the potency ratio of the constituents is constant, i.e., that the log dose-effect curves do not differ significantly from parallelism [17]. To check this assumption, we calculated the hill slopes and their CI95s of the does-effect curves. They were 1.929 (1.362~2.497), 3.503 (2.191~4.815), 3.755 (2.429~ 5.081), 1.936 (1.357~2.516), 3.139 (1.634~ 4.645), and 3.468 (2.330~4.605) for the six dose ratios, respectively. All of these CI95s were overlapped. Thus, the assumption for linear isobolographic methods was satisfied.

The experimental and theoretical ED_{50}s (Z*mix and Z*add) for each mixture ratio and their corresponding CI95s were shown in Table 1. It was clear that the CI95s of 1 mg/6.25 ng, 1 mg/12.5 ng, 1 mg/25 ng, and 1 mg/200 ng did not overlap between Z*mix and Z*add. Also, for the last three ratios, Z*mix was smaller than Z*add. Therefore, the mixture's ED_{50} was significantly lower than that expected from simple additivity. This meant that the mixtures of the two drugs with these three ratios showed clear synergistic effect. On the other hand, the combination with a ratio of 1 mg/6.25 ng showed sub-additivity according to our result. For the ratio 1 mg/100 ng, Z*mix and Z*add were marginally overlapped. Thus, with this ratio, the mixture had the tendency of super-additivity.

Table 1. Summarization of isobolographic analysis for the six combinations

TRA / DHE (mg / ng)	Experimental		Theoretical		Inference
	Z*mix	CI95	Z*add	CI95	
1/6.25	28.22	24.08~33.06	21.12	20.63~21.60	Sub-additivity
1/12.5	13.59	11.84~15.61	19.25	18.44~20.05	Synergy
1/25	12.73	11.31~14.32	16.34	15.16~17.52	Synergy
1/50	12.92	10.99~15.20	12.56	11.12~13.99	Additivity
1/100	6.335	5.316~7.55	8.582	7.188~9.974	Additivity
1/200	2.935	2.646~3.256	5.255	4.159~6.350	Synergy

TRA, tramadol; DHE, dihydroetorphine; Z*mix, experimental ED_{50} of the mixture; Z*add, theoretical ED_{50} of the mixture; CI95, 95% confidence interval. Z*mix and the related CI95s came directly from the experimental data, while Z*add and their CI95s were calculated from the does-response curves of the two drugs alone.

However, the remaining ratio 1 mg/50 ng did show additivity. The Z*mix (12.92 mg/kg) and Z*add (12.56 mg/kg) were almost the same. Thus, the two drugs displayed no synergistic effect if delivered with a ratio close to their ED_{50}s (1 mg/50 ng). They even showed sub-additivity if the proportion of DHE was too small (below 1 mg/ 6.25 ng). The isobolograph was shown in Fig. 3.

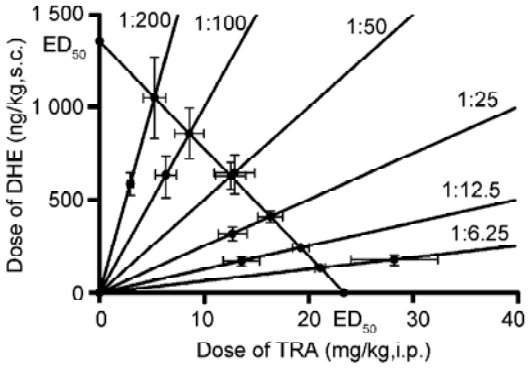

Fig. 3. Isobolograph of the TRA-DHE co-application. The results of isobolographic analysis were summarized. Numbers close to the radial lines starting from zero indicate the ratio of the binary mixture. Round dots show the experimental ED_{50}s, and the position where these radial lines cross the addition line show the theoretical ED_{50}s. TRA, tramadol; DHE, dihydroetorphine; ED_{50}, 50% effective dose.

2.3 Acute tolerance of the TRA-DHE combination

The time-effect curves of the consecutive injection of TRA, DHE or a combination were shown in Fig. 4*A*. Eighty minutes after each injection, the effect of either treatment approached the baseline. The effect decreased gradually after repeated administration of drugs, especially for TRA or DHE given alone. The MAUCs, 20~60 min after each injection, were then computed as the index of analgesic effect of each injection (Fig. 4*B*). The analgesic effect of TRA 20 mg/kg decreased after the fourth injection, and that of DHE 1 000 ng/kg alone decreased after only 3 injections. However, the effect of the combination only began to decrease after the sixth injection. These results revealed that adding a small dose of DHE (250 ng/kg) could significantly postpone the occurrence of acute tolerance to TRA analgesia.

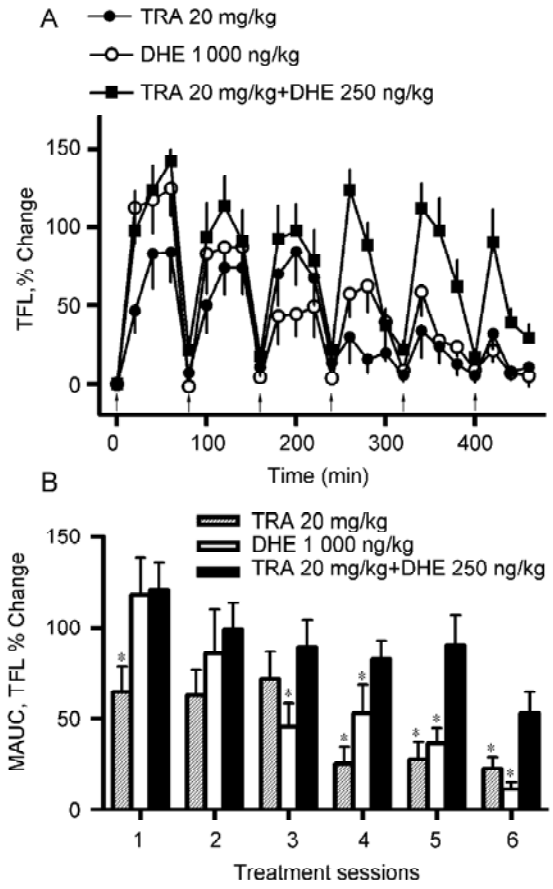

Fig. 4. Acute tolerance elicited by 6 sessions of TRA (20 mg/kg, i.p.), DHE (1 000 ng/kg, s.c.), or TRA (20 mg/kg, i.p.)+DHE (250 ng/kg, s.c.). *A*: Time course of drug effects. Arrows indicate the time of drug delivering. *B*: MAUCs after each injection of drugs. Two-way ANOVA revealed that the tolerance induced by the combination appeared later than any of the drug alone. n=8~10 in each group. $^{*}P<0.05$ compared with TRA+DHE group. TRA, tramadol; DHE, dihydroetorphine; TFL, tail-flick latency; MAUC, mean area under the curve.

3 DISCUSSION

Many reports have been published on analgesic combinations[21-23]. It has also been demonstrated that the combination of drugs acting on different receptors may produce super-or sub-additive interaction in antinociceptive effects in rats. However, there are no data yet to verify whether a short-acting drug with selective μ-opiate receptor activity and a drug act on both opiate and non-opiate pathway could produce interaction. Therefore, the focus of this study was to quantitatively evaluate the analgesic interaction in such a combination, DHE and TRA. Our result confirmed that TRA and DHE delivered together in certain range of dose ratios (i.e., 1:12.5, 1:25, 1:100, and 1:200, in mg : ng) did display synergistic potentiation of the antinociceptive effect, as long as they were not combined with approximately the ratio of their ED_{50}s (i.e., 1 mg / 50 ng), and the proportion of DHE was not too small (i.e., 1 mg : 6.25 ng).

3.1 Possible mechanisms of the observed synergy

The mechanism of the synergism between TRA and DHE is yet unknown. DHE is a selective μ-opioid receptor ligand [24]. TRA is a weak opioid agonist with antinociceptive effects through its action on μ-receptor and by inhibiting the neuronal re-uptake of both noradrenaline and serotonin. Hence, this interaction is most possibly because of the interaction between opioidergic and serotonergic or adrenergic pathways.

It has been previously demonstrated that morphine dose-dependently inhibits 5-HTP-induced head-twitch response in mice[25,26]. Interestingly, TRA also reduces the 5-HTP-induced head-twitch response in mice, presumably via the activation of μ and κ opioid receptors[27]. Further investigation reveals that opioids inhibit both local GABAergic and glutamatergic cells projecting onto dorsal raphe nucleus serotonergic neurons. The summation of these effects is to suppress inhibitory postsynaptic currents in these neurons, hence induce an increase in extracellular serotonin concentration in the rat diencephalons[28]. This increased synaptic serotonin concentration will surely be in favor of a better analgesic effect of TRA[29,30]. Therefore, we suppose that while producing analgesic effect by itself, DHE as a strong μ-receptor agonist may greatly enhance the ability of TRA to increase the extracellular concentration of 5-HT, which at last also enhance the effect of analgesia. This explanation needs to be tested in future works.

On the other hand, it is demonstrated that TRA competitively inhibits norepinephrine transporter function in the adrenal medullar cells and probably the noradrenergic neurons of the descending inhibitory system[31]. It has been reported that α_2-adrenergic receptor agonist moxonidine combined with some opioid agonists produces spinal antinociceptive synergy[32]. This spinal antinociception and its synergy with opioids were confirmed to be mediated by α_{2C} adrenergic receptor[33]. Thus, we suggest that adding DHE into the system might form a better synergy than the weak opioid TRA itself with the elevated extracellular concentration of norepinephrine.

3.2 Possible mechanism for the postponed tolerance

Our result revealed that co-injection of TRA and DHE postponed the occurrence of tolerance. It is understandable that a synergistic combination of lower doses but with similar analgesic effect, as when applying alone, should be

less vulnerable to acute tolerance. However, it is of interest to note that adding 250 ng/kg DHE to the same dose of TRA (20 mg/kg) significantly postpone the occurrence of tolerance and generate stronger analgesia.

The mechanism of this delayed tolerance remains unknown, but it might still lie within the interaction of opioidergic pathway with serotonergic and adrenergic pathways. It has been reported that F13640, a selective 5-HT_{1A} receptor agonist, caused hyperalgesia followed by analgesia[34,35]; continuous infusion of F13640 caused hyperalgesia and 'inverse' tolerance followed by minor analgesia[35]. This inverse tolerance can be explained with signal transduction theory in pain processing [36]. Thus, with the presentation of DHE, TRA may be more likely to act as serotonin transporter inhibitor and delay the tolerance via the potential 'inverse' effect. On the other hand, activation of brainstem opioid receptors is known to modulate spinal nociceptive processing also through descending adrenergic systems, specifically, via spinal α_2 receptors[37]. Activation of α_2 receptors, in turn, blocks the functional antagonism of the opioid effects mediated by the activation of NMDA receptors and up-regulation of adenylyl cyclase and nitric oxide synthase [38]. Hence, by elevating the extracellular concentration of spinal noradrenaline, TRA might actually block the mechanism of these functional antagonism to opioid analgesia, therefore attenuate possible earlier tolerance. Further investigations are necessary to clarify the mechanism of this postponed tolerance as well as the synergy reported in the current study.

3.3 Methodological concerns

Isobolographic analysis provides a fundamental basis for assessing whether biological responses induced by mixtures of agents are greater, equal or smaller than expected on the basis of the individual activities of the component agents and the concept of dose additivity[17]. The limitation of this methodology is that the log dose-effect curves should not differ significantly from parallelism. Fortunately, our data did not violate this assumption. Thus, the relationship drawn from our data can be applied to different combinations and doses.

Another concern is that there might be gender difference for the effect of the two analgesics. The conclusion would be limited if only one gender of animals were employed in an experiment. Therefore, we chose to use both male and female rats in our experiments, and no significant gender difference was observed in our current study.

It is always an easy way to calculate dose-response curve by using the maximum effect of each dose. However, the rationality of it becomes dubious when this maximum does not always happen at the same time after drug administration. Further more, since we are interested in the co-action of two drugs, using the maximum effect becomes extremely fragile when the time-courses of the two drugs do not totally overlap. Thus, we decided to use the mean area under the time-effect curve to replace the maximal analgesia as an index of drug effect, which has been proved to be useful in our analysis.

3.4 Implication of the synergistic analgesia

Co-application of low-dose of TRA and DHE might be an attractive alternative of single-agent therapy. Clinical reports in China showed that subcutaneous administration of DHE (20 ~180 μg) produces analgesia but with some side effects, including dizziness, somnolence, nausea, vomiting, constipation and shortness of breath[8]. Also, clinical evidence shows nausea, drowsiness, constipation, dizziness, and sweating in association with TRA use. Furthermore, seizures at recommended dosages in patients were reported recently[39,40]. Although these side effects are much milder compared with morphine, they still encumber the wide application of either TRA or DHE. Our result has demonstrated that the combination of TRA and DHE produces stronger analgesic effect and can postpone the occurrence of acute tolerance in rats. This means using them together might have higher efficacy that lasts longer, while possibly with less side effect. We suggest that systematic studies targeted at defining the minimum dose required in a cocktail combination that can achieve a maximum response should be merited. Together with increasing demands by medical community and patients suffering from pain, it could be foreseen that the evolution in the analgesia of drug combination will occur predominantly in the next few years [41].

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ACKNOWLEDGEMENTS: The authors wish to thank Professor QIN Bo-Yi for providing dihydroetorphine and Beijing Four-Ring Pharmaceutical Factory for providing tramadol HCl. We also wish to thank Dr. Albert C. H. Yu for language editing and many constructive suggestions.

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