Inhibition of Guiyuan tablets on morphine-induced tolerance and hyperalgesia in mice

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Abstract: **OBJECTIVE** To investigate the effect of Guiyuan tablets on the analgesic effect of morphine and morphine-induced tolerance and hyperalgesia. **METHODS** ① The model of morphine-induced acute tolerance Mice were ig treated with Guiyuan tablets 200, 400 and 800 mg·kg^{-1} and 15 min later were sc treated with morphine 10 mg·kg^{-1} every hour for consecutive 9 h. At 24 and 48 h, they were sc treated with morphine 10 mg·kg^{-1} alone, respectively. ② The model of morphine-induced chronic tolerance Mice were ig treated with Guiyuan tablets 200, 400 and 800 mg·kg^{-1} and 15 min later were sc treated with morphine 10 mg·kg^{-1} every day for consecutive 8 d. On d 9, the mice were sc treated with morphine 10 mg·kg^{-1} alone. ③ The model of morphine-induced established tolerance. Mice were sc treated with morphine 10 mg·kg^{-1} every day for consecutive 8 d. On d 1, d 4 or d 7, the mice began to be ig co-administered with Guiyuan 200 mg·kg^{-1}. On d 9, they were sc treated with morphine 10 mg·kg^{-1} alone. The hot-plate test was used to detect the values of the baseline latency (T_0) and the post-treatment latency (T_1) before the percentage of maximal possible analgesic effect (%MPAE) was calculated. Spectrophotometry was used to detect the nitric oxide synthase (NOS) activity and the nitric oxide (NO) content in the tissue of the spinal cord. **RESULTS** The ED_{50} of the analgesic effect of Guiyuan tablets was 523.5 mg·kg^{-1} in the hot-plate test. Guiyuan tablets 200 and 400 mg·kg^{-1} prolonged the duration of morphine anti-nociception and deceased its ED_{50} from 4.67 to 3.14 and 0.65 mg·kg^{-1}, respectively. In the models of both acute and chronic tolerance, Guiyuan tablets prevented the decrease of the %MPAE and the baseline latencies ($P<0.05$). In the model of morphine-induced established tolerance, Guiyuan tablets rapidly reversed the decrease of %MPAE ($P<0.05$), and this compound preparation which began to be co-administered with morphine from d 1 could significantly inhibit the increase of the NOS activity and NO content induced by morphine in the spinal cord ($P<0.01$). **CONCLUSION** Guiyuan tablets are capable of enhancing the analgesic effect of morphine, prolonging the duration of morphine anti-nociception, preventing the development of morphine-induced tolerance and hyperalgesia, and might have neuroprotective effect.

Key words: drugs, Chinese herbal; Guiyuan tablet; morphine; pain; drug tolerance; hyperalgesia
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Morphine, as a powerful analgesic, is used in clinically to relieve moderate-to-severe pain for patients, especially for those with terminal cancer. However, inappropriate use, such as repeated administration at high doses, is likely to cause many serious adverse effects including dependence, tolerance, hyperalgesia and respiratory depression[1-3], which further aggravate patients' pain. Therefore, in order to improve the life quality of patients', it's urgent to discover some potent analgesic adjuvants which are capable of enhancing the analgesic effect of morphine and preventing the development of morphine-induced dependence, tolerance and hyperalgesia.

Morphine-induced tolerance and hyperalgesia are two related but different clinical occurrences. The former means that after repeated or chronic exposure to morphine, patients need a larger dose of morphine to obtain the same analgesic

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effect, and the latter is that patients treated with morphine show a hypersensitivity to the same nociceptive stimulation[1-2]. Up to now, the neural mechanisms of the two phenomena are not completely understood, but there are some overlaps between them. One of these overlaps which has been demonstrated is the neuronal plastic changes within the spinal cord. These changes are involved in the activation of excitatory amino acid (EAA) receptors and subsequent intracellular events[2,4-8]. Glutamate is a major excitatory neurotransmitter in the central nervous system (CNS), which can activate the *N*-methyl-*D*-aspartate receptors (NMDARs) and downstream signaling events, including Ca^{2+} influx, neuronal nitric oxide synthase (NOS) activation and nitric oxide (NO) production[4,6]. NO overproduction can enhance the NMDAR-dependent signaling in a positive feedback manner, which will facilitate the development of morphine-induced tolerance and hyperalgesia by decreasing the morphine-activated μ-opioid receptor signaling[5,9].

The Guiyuan tablets is a traditional Chinese medicine compound preparation, which is mainly used in the treatment of drug addiction and consists of several medicinal herbs extracts, such as Radix Ginseng, Radix Stephaniae Epigaeae and Radix Astragali. In our previous studies, this compound preparation showed its benefits in the treatment of morphine dependence both in clinical trials and animal experiments[10-12]. Moreover, it also produced a weak-to-moderate analgesic effect in the mice hot-plate and acetic acid writhing tests[13]. However, it's unknown whether this preparation could enhance the morphine analgesia and prevent the development of morphine-induced tolerance and hyperalgesia. Therefore, in this study, the hot-plate test was used to evaluate the time-effect and the dose-effect relationships of the analgesic effect of morphine after co-administration with Guiyuan tablets. The effect of Guiyuan tablets on the morphine-induced tolerance and hyperalgesia by establishing the models of acute, chronic and established tolerance was also evaluated. In order to investigate whether the effect of Guiyuan tablets on morphine-induced tolerance and hyperalgesia is involved in the NMDARs/Ca^{2+}/nNOS/NO signaling pathway, the NOS activity and NO content in the spinal cord tissues of mice were detected.

1 MATERIALS AND METHODS

1.1 Drugs and equipment

Guiyuan tablets were prepared by Yabao Pharmaceuticla Group Co. Ltd (Shanxi, China) The dry power of Guiyuan tablets was dissolved in distilled water for intragastric administration (ig). Morphine hydrochloride (Qinghai Pharmaceutical Factory, China) obtained from Institute of Pharmacology and Toxicology was dissolved in 0.9% saline for subcutaneous injection (sc). All compounds were administered in a delivery volume of 0.01 $mL \cdot g^{-1}$. YLS-6B intelligent hot plate apparatus (Ji'nan Yi Yan Technology Development Co., Ltd., China); TE124S electronic analytical balance (Sartorius, Germany); UV-1200 spectrophotometer (Mapada, China); D2012 high speed mini centrifuge (Scilogex, USA); Kits of nitric oxide synthase activity and nitric oxide content (Nanjing Jiancheng Biocompany, Nanjing, China).

1.2 Animals

Adult female Kunming mice (18-22 g) used in this study were purchased from the Experimental Animal Center of Academy of Military Medical Sciences [Beijing, China, Certificate Number: SCXK-(Military)-2012-0004]. All animals were maintained under standard laboratory conditions and kept in a temperature and humidity controlled room (22±1)℃, (55±5)% with a 12 h light-dark cycle (lights on from 7:00 am to 7:00 pm). Food and distilled water were available ad libitum. All animal experiments were performed in accordance with the guidelines of the Academy of Military Medical Sciences Animal Care and Use Committee.

1.3 Hot-plate test for detection of baseline and post-treatment latencies

The analgesic effect of drugs was determined using the hot-plate test. Nociceptive latency of mice was defined as the response time that animals took to lick its hindpaws or jump with all four feet to the hot plate (55℃). The maximal time of mice exposure to the hot plate was artificially imposed as 60 s to avoid tissue damage. For each mouse, the baseline latency and post-treatment latency were determined before and after drug administration, respectively. The result was expressed as the percentage of maximal possible analgesic effect (%MPAE). %MPAE = (post-treatment latency − baseline latency)/(60 s − baseline latency) × 100%.

1.3.1 Detecting the analgesic effect of Guiyuan tablet

Naïve female mice were randomly divided into 5 groups with 10 mice each, including the normal control, morphine (5 mg·kg^{-1}), and Guiyuan tablet (200, 400 and 800 mg·kg^{-1}) groups. The post-treatment latencies at a serial time points of 0, 30, 60, 90, 120, 180, 240 and 300 min were tested to observe the time-effect relationship of Guiyuan tablet analgesia. Other naïve female mice were also randomly divided into 5 groups with 10 mice each, which were treated with Guiyuan tablets at the doses of 100, 200, 400, 800 and 1600 mg·kg^{-1}, respectively. Then the dose-effect relationship of Guiyuan tablet analgesia was assessed by fitting the dose-response curve and calculating the ED_{50} value.

1.3.2 Detecting the analgesic effect of morphine and Guiyuan tablets

Naïve female mice were randomly divided into three groups with ten mice each. They were the groups of morphine (5 mg·kg^{-1}), and morphine combined with Guiyuan tablets 200 and 400 mg·kg^{-1}, respectively. The time-effect relationship of the analgesic effect of morphine combined with Guiyuan tablets was observed by detecting the post-treatment latencies at a serial time points of 5, 20, 35, 50, 65, 95, 125, 185, and 305 min.

Other naïve female mice were also randomly divided into three groups which were subdivided into six subgroups. These groups included the group of morphine at a serial doses of 2.7, 3.5, 4.5, 5.9, 7.7 and 10 mg·kg^{-1} (ten mice for each dose), the group of Guiyuan tablets (200 mg·kg^{-1}) combined with morphine at a serial doses of 1, 1.5, 2.3, 3.5, 5.3 and 8 mg·kg^{-1} (six mice for each dose), and the group of Guiyuan tablets (400 mg·kg^{-1}) combined with morphine at a serial doses of 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 mg·kg^{-1} (ten mice for each dose). The dose-effect relationship of the analgesic effect of morphine or morphine plus Guiyuan tablets was assessed by fitting the dose-response curves and calculating the ED_{50} values.

1.3.3 Morphine-induced acute tolerance

Naïve female mice were randomly divided into five groups with ten animals each, including normal control, morphine, and morphine combined with Guiyuan tablets (200, 400 and 800 mg·kg^{-1}) groups. Animals were pretreated with distilled water or Guiyuan tablets (ig), and 15 min later were subcutaneously injected with saline or morphine (10 mg·kg^{-1}), which were performed once per hour for consecutive 9 h. Twenty-four and forty-eight hours after the first administration, except for the normal control group, each group was treated with morphine 10 mg·kg^{-1} (sc) alone. At the time points of 0, 9, 24 and 48 h, the baseline (or pre-treatment) latencies and the post-treatment latencies were tested and recorded, respectively.

1.3.4 Morphine-induced chronic tolerance

Naïve female mice were randomly divided into five groups with ten animals each, including normal control, morphine and morphine combined with Guiyuan tablets (200, 400 and 800 mg·kg^{-1}) groups. Animals pretreated with distilled water or Guiyuan tablets (ig) were subcutaneously treated with saline or morphine (10 mg·kg^{-1}) once a day for eight days. On the 9th day (d 9) except for the normal control group, each group was treated with morphine (10 mg·kg^{-1}; sc) alone. The baseline and the post-treatment latencies were tested and recorded daily.

1.3.5 Morphine-induced established tolerance

Naïve female mice were randomly divided into six groups with ten animals each, including normal control, Guiyuan tablets, morphine, and morphine combined with Guiyuan tablets from d 1, d 4 or d 7 groups. The mice were given morphine (10 mg·kg^{-1}; sc) for 8 d, and began to be co-administered with Guiyuan tablets (200 mg·kg^{-1}; ig) from d 1, d 4 and d 7, respectively. On the d 9, except for the normal control group, each group was treated with morphine 10 mg·kg^{-1} alone. The baseline latency and the post-treatment latency were tested and recorded daily.

1.4 Detection of NOS activity and NO content in spinal cord of morphine-induced established tolerance mice

In the model of morphine-induced established tolerance, all animals were sacrificed by cervical luxation 60 min after the last administration. The spinal cords were removed quickly on a ice-plate washed with cold saline, blotted with filter paper and stored at −20℃ until used. The NOS activity and the NO content in the spinal cord were detected at 24 h after sample collection in accordance with the instructions of their commercially available kits.

1.5 Statistical analysis

The data were expressed as $\bar{x}\pm s$, and were assessed using one-way ANOVA followed by

Dunnett′s multiple comparison (SPSS 18.0 software, Chicago, IL, USA) or using two-way ANOVA followed by Bonferroni′s multiple comparison (GraphPad Prism 5.0 Software, San Diego, CA, USA). The level for statistical significance was $\alpha=0.05$ and the statistical charts were drawn by GraphPad 5.0 Software.

2 RESULTS

2.1 Analgesic effect of Guiyuan tablets in hot-plate test

As shown in Fig. 1, Guiyuan tablets had a weak-to-moderate analgesic effect and had an ED_{50} value of 523.5 mg·kg^{-1} (95%CI: 362.2 to 756.8). Sixty minutes after ig administration, the %MPAE values of Guiyuan tablets at the doses of 200, 400 and 800 mg·kg^{-1} were (22.49±6.18)%, (52.04±12.49)% and (78.57±10.58)%, respectively. Compared with the normal control group [%MPAE = (3.17±1.55)%], Guiyuan tablets 200 mg·kg^{-1} group didn′t showed significant analgesic effect. The analgesic effect of Guiyuan tablets 800 mg·kg^{-1} was similar to that of morphine 5 mg·kg^{-1} [%MPAE = (70.84±12.98)%]. Moreover, the duration of the analgesic effect of Guiyuan tablets was longer than that of morphine.

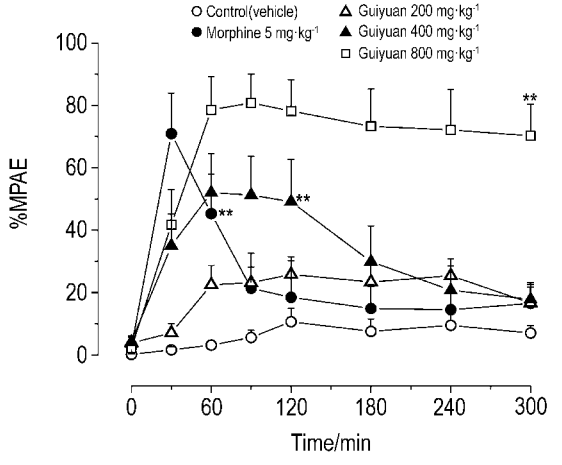

Fig.1 Analgesic effect of Guiyuan tablets in hot-plate test. %MPAE: the percentage of maximal possible alalgesic effect. $\bar{x}\pm s$, $n=10$. $^{**}P<0.01$, compared with the normal control group.

2.2 Effect of Guiyuan tablets on morphine analgesia in hot-plate test

As shown in Fig. 2, Guiyuan tablets could markedly prolong the duration of morphine analgesia and decreased its ED_{50} value. 95 min after administration, the analgesic effect of morphine (5 mg·kg^{-1}) alone waned to (11.2±9.9)%, whereas morphine plus Guiyuan tablets (200 and 400 mg·kg^{-1}) still exhibited marked analgesic effect with the %MPAE of (79.7±11.0)% and (97.2±2.8)%, respectively. These effects of the two co-administration groups could be seen even up to 305 min after administration with the %MPAE of (51.6±12.8)% and (52.5±11.3)%, respectively (Fig.2A). Moreover, Guiyuan tablets at the doses of 200 and 400 mg·kg^{-1} decreased the ED_{50} value of morphine analgesia from 4.67 mg·kg^{-1} (95%CI: 3.87 to 5.64) to 3.14 mg·kg^{-1} (95%CI: 1.95 to 5.06) and 0.65 mg·kg^{-1} (95%CI: 0.40 to 1.06), respectively (Fig.2B).

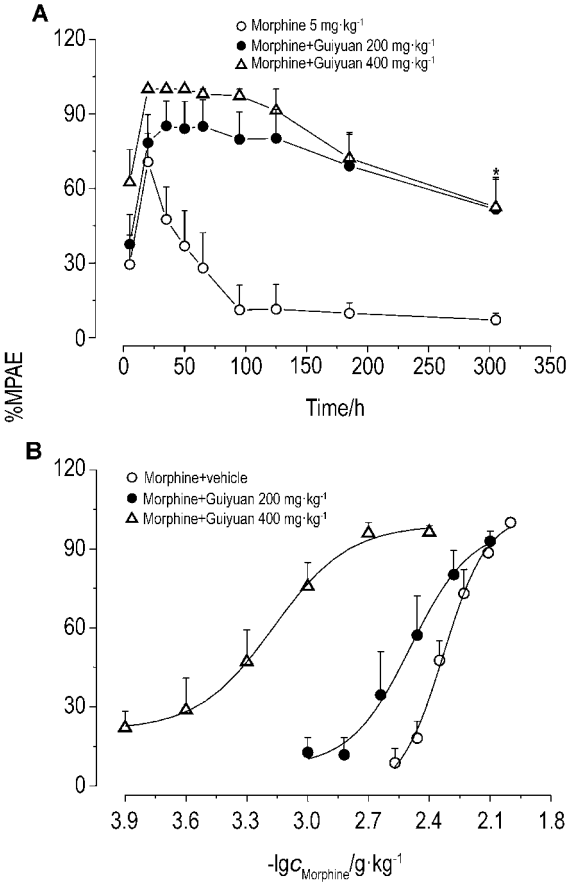

Fig.2 Effect of Guiyuan tablets on morphine analgesia detected by hot-plate test. Animals were ig treated Guiyuan tablets 15 min prior to 5 mg·kg^{-1} morphine (sc). $\bar{x}\pm s$, $n=6-10$. $^{*}P<0.05$, compared with the morphine group.

2.3 Effect of Guiyuan tablets on the morphine-induced acute tolerance and hyperalgesia

The data revealed that after repeated administrations for consecutive 9 h, the analgesic effect of morphine declined markedly, but Guiyuan tablets showed a dose-dependent inhibition on this decreasing tendency. The %MPAE values of

morphine combined with Guiyuan tablets 200, 400 and 800 mg·kg^{-1} groups were (90.1±6.8)%, (96.6±3.4)% and 100%, respectively, which were significantly higher than those of morphine group [(51.9±14.6)%, $P<0.01$]. This inhibitory effect of Guiyuan tablets even lasted to 24 and 48 h. Forty-eight hours after the first administration, the %MPAE values of the groups of morphine combined with Guiyuan tablets 200, 400 and 800 mg·kg^{-1} were (59.9±9.5)%, (60.1±11.5)% and (60.0±11.4)%, respectively, which were still significantly higher than those of the normal control [(3.1±3.0)%, $P<0.01$] and the morphine [(22.3±6.2)%, $P<0.05$] groups (Fig.3A). Although the baseline latency of morphine group was significantly decreased from the original (16.9±4.4) s to (10.7±2.6) s before the last administration (t=3.516, $P<0.01$), that of the morphine plus Guiyuan tablet groups didn't show any significant decrease ($P>0.05$; Fig.3B).

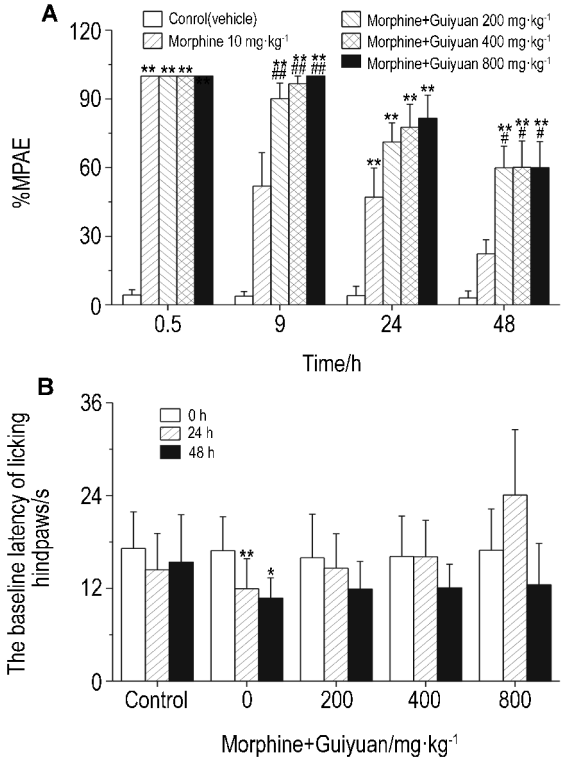

Fig.3 Effect of Guiyuan tablets on morphine-induced acute tolerance (A) and hyperalgesia (B). $\bar{x}\pm s$, n=10. $^{*}P<0.05$, $^{**}P<0.01$, compared with the normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the morphine group of Fig.A. $\bar{x}\pm s$, n=10. $^{*}P<0.05$, $^{**}P<0.01$, compared with each corresponding group at the time point of 0 h of Fig.B.

2.4 Effect of Guiyuan tablets on morphine-induced chronic tolerance and hyperalgesia

As shown in Fig.4, after repeated administrations, the %MPAE value of morphine group decreased from 100% on day 1 to (24.7±9.1)% on day 8, but the values of morphine plus Guiyuan tablets (200, 400 and 800 mg·kg^{-1}) groups were just reduced to (59.5±10.8)%, (80.7±7.9)% and (83.4±8.8)%, respectively. On day 9, except for the normal control group, each group was treated with morphine alone. The %MPAE values of the three co-administration groups were (60.2±10.7)%, (57.9±6.3)% and (59.4±8.6)%, respectively, significantly higher than those of either the normal control group [(2.6±0.9)%, $P<0.01$] or the morphine group [(20.6±9.4)%, $P<0.01$] (Fig.4A). Although the baseline latencies of the normal control and morphine groups significantly decreased from (18.2±5.0) s and (19.3±5.9) s on d 1 to (8.9±3.2) s and (12.4±4.7) s on d 9, respectively (the normal control group: t=5.201, $P<0.01$; the morphine group: t=3.810, $P<0.01$), that of the three morphine combined with Guiyuan tablets groups did not show any notable decrease (Fig.4B).

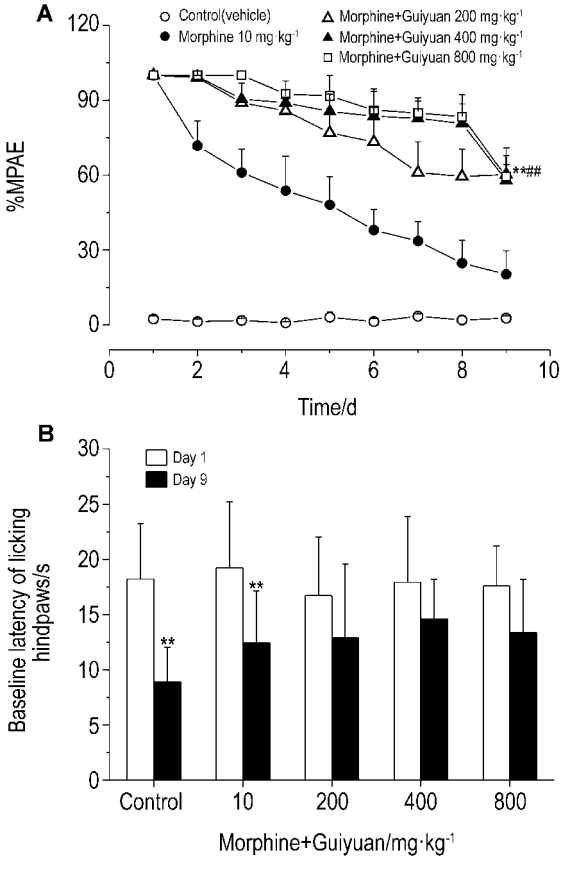

Fig.4 Effect of Guiyuan tablets on morphine-induced chronic tolerance (A) and hyperalgesia (B). $\bar{x}\pm s$, n=10. $^{**}P<0.01$, compared with the normal control group; $^{\#\#}P<0.01$, compared with the morphine group of Fig.A. $\bar{x}\pm s$, n=10. $^{**}P<0.01$, compared with each corresponding group on d 1 of Fig.B.

2.5 Effect of Guiyuan tablets on morphine-induced established tolerance and hyperalgesia

The results suggested that Guiyuan tablets at the dose of 200 mg · kg^{-1} with no significant analgesic effect could effectively eliminate the development of morphine tolerance when co-administered from d 1, and could quickly reverse the morphine-induced established tolerance when co-administered from d 4 or d 7. On d 9, when animals were treated with morphine alone, morphine group with a %MPAE value of (22.8±9.3)% did not show any preponderance in analgesia, but the groups of morphine plus Guiyuan tablets from d 1, d 4 or d 7 with their respective %MPAE values of (47.7±13.0)%, (54.3±10.0)% and (44.9±7.2)% still showed a significant analgesic effect [the normal control group: %MPAE = (2.2±1.0)%, $P<0.01$] (Fig.5A). When Guiyuan tablets(200 mg·kg^{-1}) began to be co-administered from d 1, they effectively inhibited the decrease in the baseline latency induced by morphine [d 1: (19.38±3.07)s; d 9: (14.3±7.4)s; $t=2.727$, $P>0.05$], but failed to reverse this decrease when it began to be co-administered from d 4 [d 1: (19.71±4.02)s; d 9: (7.92±2.36)s; $t=6.366$, $P<0.01$] or d 7 [d 1: (19.82±3.39)s; d 9: (10.22±4.77)s; $t=5.187$, $P<0.01$] (Fig.5B).

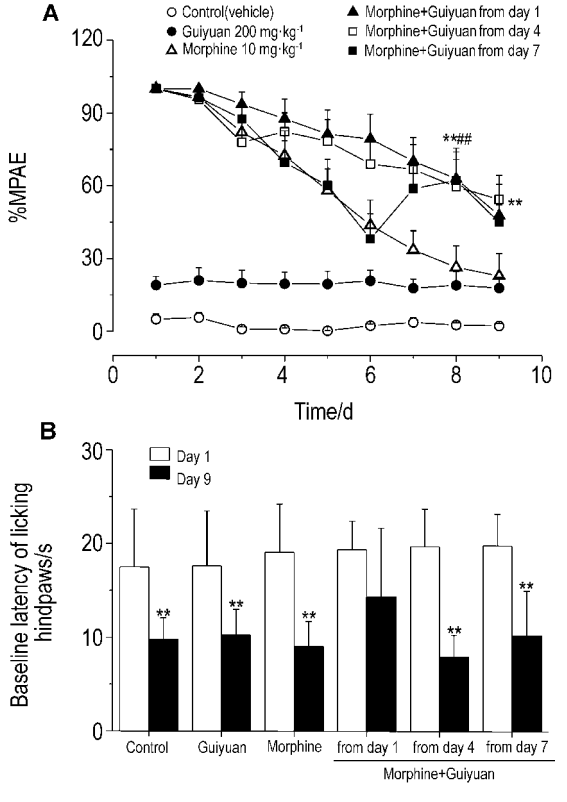

Fig.5 Effect of Guiyuan tablets (200 mg · kg^{-1}) on morphine-induced established tolerance (A) and hyperalgesia (B). $\bar{x}\pm s$, $n=10$. $^{**}P<0.01$, compared with the normal control group; $^{\#\#}P<0.01$, compared with the morphine group of Fig.A. $\bar{x}\pm s$, $n=10$. $^{**}P<0.01$, compared with each corresponding group from day 1 of Fig.B.

2.6 Effect of Guiyuan tablet on NOS activity and NO content in spinal cord of morphine-induced established tolerance mice

As shown in Tab.1, Guiyuan tablets 200 mg·kg^{-1}, (ig) alone had no effect on the levels of these two biochemical parameters, but morphine 10 mg·kg^{-1}, (sc) caused an increase both in the NOS activity ($P<0.01$) and in the NO content ($P<0.01$), which was effectively blocked by Guiyuan tablets. Especially for the co-administration from d 1, the levels of NOS activity and NO content were significantly decreased by Guiyuan tablets [NOS activity: (7.24±0.36) kU · g^{-1} protein, and NO content: (1.38±0.09) μmol · g^{-1} protein; $P<0.05$], respectively.

Tab.1 Effect of Guiyuan tablets on nitric oxide synthase (NOS) activity and nitric oxide (NO) content in the spinal cord of morphine-induced established tolerance mice

Group	NOS activity/ kU·g^{-1} protein	NO content/ μmol·g^{-1} protein
Normal control	7.09±0.34	1.24±0.15
Guiyuan 200 mg·kg^{-1}	6.97±0.38$^{\#\#}$	1.23±0.15$^{\#\#}$
Morphine 10 mg·kg^{-1}	7.93±0.37**	1.79±0.11**
Morphine+Guiyuan from day 1	7.24±0.36$^{\#\#}$	1.38±0.09$^{\#\#}$
day 4	7.53±0.34	1.48±0.12$^{**\#\#}$
day 7	7.70±0.36**	1.53±0.13$^{**\#\#}$

$\bar{x}\pm s$, $n=10$. $^{*}P<0.05$, $^{**}P<0.01$, compared with the normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the morphine group.

3 DISCUSSION

The findings of the present study showed that Guiyuan tablets were able to enhance the analgesic effect of morphine, reflected by the decreased ED_{50} value and the prolonged duration. Meanwhile, Guiyuan tablets not only effectively prevented the development of morphine-induced acute and chronic tolerance, but also quickly reversed the morphine-induced established tolerance. In addition, the obtained results indicated that this compound preparation could prevent the development of morphine-induced acute and chronic hyperalgesia, but failed to reverse the established hyperalgesia. *In vitro*, Guiyuan tablets had no influence on the levels of NOS activity and NO content in the spinal cord of animals, but could inhibit

their increased levels induced by chronic morphine exposure when this compound preparation was co-administered from d 1. However, when co-administered from d 4 – d 7, this preparation failed to reverse the formed high levels of NOS activity and NO content induced by morphine.

In this study, Guiyuan tablets exhibited a pharmacological profile of weak-to-moderate antinociception with an ED_{50} value of 523.5 $mg \cdot kg^{-1}$ in the mice hot-plate test, which was consistent with our previous observation[13]. Guiyuan tablets had no or weak analgesic effect at the dose of 200 or 400 $mg \cdot kg^{-1}$, but when it was co-administered with morphine, there was a marked leftward shift in the dose-response curve of morphine antinociception. At the same time, our data showed that the %MPAE values of Guiyuan tablets (200 $mg \cdot kg^{-1}$) and morphine (5 $mg \cdot kg^{-1}$) were 15%–20% and 60%–70%, respectively, and the value of their co-administration was 80% – 85%, suggesting that there might be a synergistic analgesic action between morphine and Guiyuan tablets. Besides, Guiyuan tablets could significantly prolong the duration of morphine anti-nociception, which might be independent on the analgesic effect of Guiyuan tablets, for the preparation at the dose of 200 $mg \cdot kg^{-1}$ had no analgesic effect, but significantly prolonged the duration of the effective anti-nociception of morphine 5 $mg \cdot kg^{-1}$ from 65 min to 305 min. However, it′s unclear why Guiyuan tablets could increase the duration of morphine analgesia. A large amount of evidence demonstrated that the analgesic effect of morphine including intensity and duration was closely related to its metabolism *in vivo*, especially for its plasma concentration[14-17]. So the effect of Guiyuan tablets on the duration of morphine analgesia might be associated with its inhibition on morphine metabolism in the body. Unfortunately, we didn′t detect the plasma concentration of morphine after administration in this study. In order to identify the mechanism of the prolonged effect of this compound preparation on the duration of morphine analgesia, further research is needed to observe the pharmacokinetic profile of morphine when administered alone or co-administered with Guiyuan tablets.

It′s known that chronic or repeated exposure to morphine can easily induce tolerance and hyperalgesia[2]. The results of our study revealed that Guiyuan tablets could prevent the development of morphine-induced acute and chronic tolerance effectively. This inhibitory effect was not dose-dependent, because either in the acute or in the chronic tolerance model, after the last administration with morphine alone, the %MPAE values of the groups of morphine plus Guiyuan tablets at different doses were similar. In the model of morphine-induced established tolerance, meanwhile, Guiyuan tablets were capable of reversing the established tolerance rapidly. Based on these findings, Guiyuan tablets indeed contributed to antagonizing the morphine-induced tolerance. Moreover, our data also showed that this preparation could attenuate the development of the morphine-induced acute and chronic hyperalgesia, but failed to reverse the established hyperalgesia induced by morphine. What was mentioned above not only suggested that in order to effectively prevent the development of morphine-induced tolerance and hyperalgesia, Guiyuan tablets should be co-administered with morphine from the beginning in clinical pain management, but also indicated that there might be some different mechanisms between the effects of Guiyuan tablets on morphine-induced tolerance and hyperalgesia.

Morphine-induced tolerance and hyperalgesia are two different adverse effects, but they are closely associated with each other and share some molecular mechanisms[2]. Among their overlapping mechanisms, the neuronal plastic changes within the spinal cord have been considered an important one, which involve the activation of excitatory amino acid receptors and the transduction of subsequent signaling pathway such as NMDARs/Ca^{2+}/nNOS/NO[4-6,19-21]. Therefore, in order to determine whether the inhibitory effect of Guiyuan tablets on morphine-induced tolerance and hyperalgesia was involved in the neuronal plastic changes induced by chronic morphine exposure, we detected the levels of NOS activity and NO content in the spinal cord of mice in the present study. Our data revealed that chronic morphine administration indeed increased the levels of NOS activity and NO content in the animal spinal cord significantly as previously studies[4,9], and Guiyuan tablets had no influence on the levels of the two biochemical parameters. When this preparation began to be co-administered with morphine from d 1, it could significantly inhibit the increase of NOS activity and NO content induced by morphine, which suggested that Guiyuan tablets

potentially confer neuroprotection that might be attributed to the mechanisms of the inhibitory effect of Guiyuan tablets on morphine-induced tolerance and hyperalgesia.

It is worth noting that when Guiyuan tablets began to be co-administered with morphine from d 4 or d 7, they failed to effectively reverse the high levels of NOS activity or NO content induced by morphine. This result was consistent with the behavioral effect of Guiyuan tablets on morphine-induced established hyperalgesia. So the molecular mechanism of Guiyuan tablets on morphine-induced hyperalgesia may be directly associated with the regulating action on the high levels of NOS activity and NO content in the spinal cord induced by morphine.

In view of the inconsistent results that Guiyuan tablets were capable of reversing the morphine-induced established tolerance but failed to reverse the formed high levels of NOS activity and NO content induced by morphine, we speculate that the inhibitory effect of Guiyuan tablets may be not directly involved in regulating the abnormal levels of NOS activity and NO content in the spinal cord. It′s known that the activation of NMDA receptors not only induces the increase of NOS activity and NO content, but also leads to the activation and translocation of protein kinase C (PKC). The latter will further mediate the morphine-activated μ-opioid receptor phosphorylation on the cell membrane. The phosphorylated μ-opioid receptor mediated by PKC shows desensitization and fails to induce endocytosis, which has been considered to be a molecular basis of the tolerance to morphine[2-3]. Therefore, besides the regulation of Guiyuan tablets on the morphine-induced high levels of NOS activity and NO content, this compound preparation may be involved in the NMDARs/Ca^{2+}/PKC pathway. We didn′t detect the levels of PKC in the present study, for the hypothesis that the inhibitory effect of Guiyuan tablets on morphine-induced tolerance is associated with the NMDARs/Ca^{2+}/PKC signaling pathway, further study is needed.

As mentioned in the introduction, Guiyuan tablets consist of several medicinal herbs extracts. The components of this preparation are so complex that it′s difficult to find out about the main bioactive constituents. In this compound preparation, Stephaniae Epigaeae as one of the components is rich in tetrahydroprotoberberines (THPBs) which belong to the isoquinoline alkaloids. Many studies have demonstrated that these alkaloids mainly act on the dopaminergic system in the brain and the spinal cord[7-9,14-23]. *l*-Tetrahydropalmatine (*l*-THP) and *l*-stepholidine (*l*-SPD) are two important active substances of THPBs, which have shown some benefits in the treatment of weak-to-moderate pain and drug addiction[23-24]. So the analgesic effect of Guiyuan tablets and the antagonism on morphine addiction may be associated with the pharmacological action of these isoquinoline alkaloids. However, whether the inhibition of Guiyuan tablets on morphine-induced tolerance and hyperalgesia is also the pharmacological properties of THPBs is far from clear. Due to the complex mechanisms of the morphine-induced tolerance and hyperalgesia, and the diversity of Guiyuan tablet composition, more efforts are needed to analyze the bioactive ingredients of this preparation and to explore the molecular mechanisms of pharmacological actions.

Moreover, we found that the baseline latencies of the normal control group did not show any obvious decrease in the model of morphine-induced acute tolerance, but they did in the model of morphine-induced chronic tolerance. This thermal-induced hyperalgesia might have been a learning process for animals repeatedly exposed to nociceptive stimuli. In the model of morphine-induced established tolerance, the baseline latency of Guiyuan tablet group was significantly decreased in the normal control group, which suggested that this preparation did not impact the learning course of animals to escape nociceptive stimuli. However, when it began to be co-administered with morphine from d 1, this preparation could effectively prevent the decrease of the baseline latencies of animals. This indicated that Guiyuan tablets could inhibit the development of the abnormal pain sensitivity induced by morphine.

In summary, our work revealed that Guiyuan tablets possessed some benefits in increasing the intensity and the duration of morphine anti-nociception and preventing the development of morphine-induced tolerance and hyperalgesia. They could effectively inhibit the increase of the NOS activity and NO content in the spinal cord induced by chronic morphine exposure, which indicated that it might have neuroprotective effect. And this neuroprotection may be involved in the mechanisms of the inhibitory effect of Guiyuan tablets on

morphine-induced tolerance and hyperalgesia. In the light of these, Guiyuan tablets have the potential to be an analgesic adjuvant to reduce the therapeutic dose of morphine, to prolong the duration of morphine anti-nociception, and to prevent the development of morphine-induced dependence, tolerance and hyperalgesia.

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归元片对小鼠吗啡耐受和痛觉过敏的抑制效应

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摘要: 目的　评价归元片对吗啡镇痛以及吗啡所致耐受和痛觉过敏的影响。**方法**　① 吗啡急性耐受模型　小鼠每隔 1 h 分别 ig 给予归元片 200,400 和 800 mg·kg^{-1},15 min 后 sc 给予吗啡 10 mg·kg^{-1},连续给 9 h,分别于 24 和 48 h 再给予吗啡 10 mg·kg^{-1};② 吗啡慢性耐受模型　小鼠每日 ig 给予归元片 200, 400 和 800 mg·kg^{-1},15 min 后 sc 给予吗啡 10 mg·kg^{-1},连续给 8 d,d 9 单独 sc 给予吗啡 10 mg·kg^{-1};③ 吗啡已形成耐受模型　小鼠每日 sc 给予吗啡 10 mg·kg^{-1} 1 次,连续给 8 d,分别于 d 1,d 4 或 d 7 起联合给予归元片 200 mg·kg^{-1},d 9 再单独 sc 给予吗啡 10 mg·kg^{-1}。采用小鼠热板测定给药前痛阈值(T_0)和给药后痛阈值(T_1),计算最大可能镇痛率(%MPAE)。采用分光光度法检测脊髓中一氧化氮合酶(NOS)活性和一氧化氮(NO)含量。**结果**　热板实验中归元片镇痛效应的 ED_{50} 为 523.5 mg·kg^{-1}。归元片 200 和 400 mg·kg^{-1}能延长吗啡镇痛时间,并使吗啡镇痛 ED_{50} 值从 4.67 mg·kg^{-1}分别降至 3.14 和 0.65 mg·kg^{-1}。在吗啡急性和慢性耐受模型中,归元片能抑制%MPAE 值和给药前痛阈值的降低(P<0.05);在已形成的耐受模型中,归元片能快速逆转已降低的%MPAE 值(P< 0.05),且当该复方制剂于 d 1 起与吗啡联用时,能有效抑制吗啡所致脊髓中 NOS 活性和 NO 含量的升高(P<0.01)。**结论**　归元片能有效增强吗啡镇痛,延长吗啡镇痛的持续时间,抑制吗啡所致耐受和痛觉过敏的发展,并可能具有神经保护作用。

关键词: 中草药; 归元片; 吗啡; 疼痛; 药物耐受性; 痛觉过敏

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