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Effects of koumine, an alkaloid of *Gelsemium elegans* Benth., on inflammatory and neuropathic pain models and possible mechanism with allopregnanolone

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ABSTRACT

Crude alkaloidal extraction from *Gelsemium elegans* Benth. produces analgesic property. However, its clinical utility has been obstructed by its narrow therapeutic index. Here, we investigated the potential of koumine, a monomer of *Gelsemium* alkaloids, to reduce both inflammatory and neuropathic pain. Interestingly, allopregnanolone, a neurosteroid, appeared to mediate the reduction of neuropathic pain. The potential anti-inflammatory pain effects of koumine were evaluated by acetic acid-, formalin- and complete Freund's adjuvant (CFA) -induced nociceptive behaviors in mice. Chronic constriction injury (CCI) and L5 spinal nerve ligation (L5 SNL), inducing thermal hyperalgesia and mechanical allodynia in rats, were used to test whether repeated treatment of koumine ameliorated neuropathic pain. Finally, we explored if koumine altered the level of neurosteroids in rat spinal cord of CCI neuropathy using liquid chromatography–tandem mass spectrometry. Koumine dose-dependently reduced the acetic acid-induced writhes and formalin-induced licking/biting time of Phase II in mice. Repeated administrations of koumine also dose-dependently reversed the CFA-, CCI- and L5 SNL-induced thermal hyperalgesia, as well as, CCI- and L5 SNL-induced mechanical allodynia in rats. The level of allopregnanolone, but not pregnenolone, in the L5-6 spinal cord was elevated by repeated treatment of koumine in CCI-induced neuropathic rats. These results demonstrate that koumine has a significant analgesic effect in rodent behavioral models of inflammatory and neuropathic pain, and that the reduction in neuropathic pain may be associated with the upregulation of allopregnanolone in the spinal cord.

1. Introduction

Chronic pain is a major health issue that presents a considerable social and economic burden worldwide. Conventionally, both inflammatory and neuropathic pains are considered the most common forms of chronic pain. Current medications for chronic pain relief primarily encompass opioid analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs), antidepressants, and anticonvulsants. Unfortunately, these medications usually provide unsatisfactory and inconstant results and produce myriad side effects, underscoring the need for novel analgesics.

Medicinal plants are an important, yet often overlooked, source for novel therapeutic drugs (Balunas and Kinghorn, 2005). Indeed, there are numerous plants that produce analgesic activity, and many botanical medicines have proven effective for relieving various forms of pain (Zareba, 2009). Thus, an increasing number of scientists have focused attention toward discovering novel analgesics from botanicals.

Gelsemium, a small genus of the family Loganiaceae, comprises three popularly known species: *Gelsemium elegans* Benth., which is native to China and Southeast Asia, and *Gelsemium sempervirens* Ait. and *Gelsemium rankinii* Small, which are native to North America (Dutt et al., 2010; Ornduff, 1970). *G. elegans* Benth. is a well-known plant in Asia, and although it can be toxic, it has been used in Chinese folk medicine as a remedy for pain, inflammation and cancer, etc. Alkaloids constitute the primary active molecules of *G. elegans* Benth. In fact, the parenteral solution of crude alkaloidal extraction has shown promise as an analgesic (Chen, 1984; Tan et al., 1988) and anti-inflammatory (Xu et al., 1991) agent in clinical research or in vivo, as well as an anti-tumor (Lu et al., 1990) agent in vitro . However, the extensive clinical use of the crude *Gelsemium* alkaloidal extraction has been greatly hampered by its narrow therapeutic index.

Currently, approximately 49 alkaloids have been isolated from *G. elegans* Benth. (Dutt et al., 2010). All of the alkaloids are similar in structure, but diverse in their pharmacological action and toxicity. Noticeably, koumine (molecular formula: $C_{20}H_{22}N_2O$; molecular weight: 306.40; CAS registry number: 1358-76-5. See Fig. 1) is the

Abbreviations: CCI, chronic constriction injury; CFA, complete Freund's adjuvant; HPLC–MS, High Performance Liquid Chromatography–Mass Spectrometry; MWL, mechanical withdrawal latency; SNL, spinal nerve ligation; TWL, thermal withdrawal latency.

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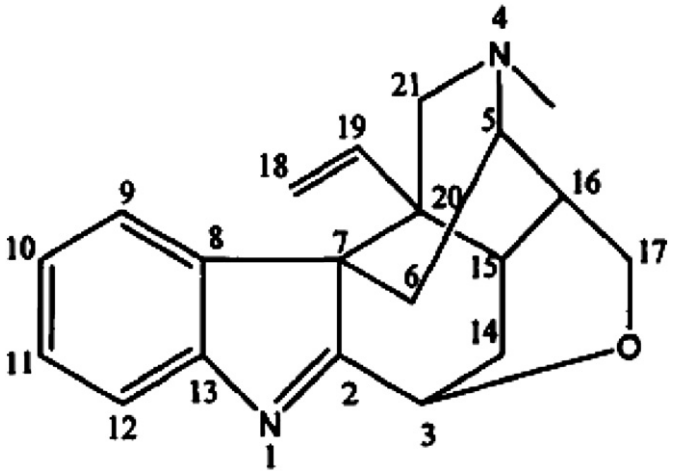

Fig. 1. Chemical structure of koumine. Molecular formula is $C_{20}H_{22}N_2O$. Molecular weight is 306.40. CAS registry number is 1358-76-5.

most abundant molecule among the alkaloids of *G. elegans* Benth. (Liu et al., 2008; Zhang et al., 2003). Excitingly, the toxicity of koumine is relatively low in comparison to the other alkaloids (Zhang et al., 2004). Therefore, there has been an increasing interest to investigate the pharmaceutical potential of koumine (Cai et al., 2007, 2009; Chi et al., 2003, 2004, 2007; Su et al., 2011; Wang et al., 2005, 2009; Wu et al., 2006; Zhang et al., 2005). For example, the antitumor (Cai et al., 2009; Chi et al., 2007; Wang et al., 2009; Wu et al., 2006), anti-stress (Cai et al., 2007) and anti-psoriasis (Zhang et al., 2005) activities of koumine have been reported. However, it has yet to be determined whether koumine has therapeutic relevance for both inflammatory and neuropathic pains. Koumine belongs to indole alkaloids, which is structurally distinct from all types of currently used analgesic medications. Moreover, koumine seems to be devoid of troublesome side effects of current medications (Dutt et al., 2010; Tan et al., 1988). As a result, its analgesic profiles and the underlying mechanism might be significantly different.

A growing body of evidence indicates that neurosteroids could modulate chronic pain (Mensah-Nyagan et al., 2008; Meyer et al., 2008, 2010, 2011; Poisbeau et al., 2005). It has been reported that gelsemine could stimulate allopregnanolone biosynthesis (Venard et al., 2011). Gelsemine also belongs to indole *Gelsemium* alkaloids. Considering the structural similarity, we hypothesized that the analgesic effects of koumine could be attributed to modulation of neurosteroids.

In the present study, we investigated the effects of koumine in behavioral models of both inflammatory [acetic acid-, formalin- and complete Freund's adjuvant (CFA) -induced] and neuropathic [chronic constriction injury (CCI) and L5 spinal nerve ligation (L5 SNL)] pains. Here we provide evidence for the first time that koumine can generate profound relief of inflammatory and neuropathic pain, and that the analgesic effect of neuropathic pain is likely associated with the upregulation of allopregnanolone in the spinal cord.

2. Material and methods

2.1. Animals, compounds, and dosing

Male ICR mice and male Sprague–Dawley rats (Shanghai Laboratory Animal Center, Chinese Academy of Sciences), weighing 18–22 g and 180–200 g respectively were used throughout the study. The animals were housed in 6–7 groups and had free access to standard rodent food and water except during behavioral experiments. Rodents were kept in a temperature-controlled room (25 ± 2 °C) with a 12-h light/12-h dark cycle (lights on from 08:00 to 20:00 h). All rodents were acclimatized for at least 1 week prior to experiments. All behavioral experiments were performed between 09:00 and 17:00 h. Each rodent was used in only one experiment. All experimental protocols were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985) and were approved by the Committee of Ethics of Fujian Medical University, China. All procedures were carried out in compliance with the guidelines for animal care and use in Fujian Medical University, China. For each experiment, animals were assigned into koumine treated groups, positive control group (except for neurosteroid determination), vehicle negative control group, and sham or naive control group (except for the writhing and formalin tests). Each experimental group consisted of at least six animals. Animals were habituated to the test room and apparatus for 30 min immediately prior to each nociceptive test. The observer scoring the behaviors was blind to drug pretreatments in all behavioral tests.

Koumine was isolated from *G. elegans* Benth. using our previously established method of pH-zone-refining counter-current chromatography (Su et al., 2011), which is capable of obtaining a purity of 99%. Morphine hydrochloride (Northeast General Pharmaceutical Factory, Shenyang, China), aspirin (Shanghai New Asiatic Pharmaceutical, Shanghai, China), indomethacin (Shanghai Xinyi Jiufu Pharmaceutical Co., Ltd, Shanghai, China), and gabapentin (purity: 99%, Shanghai Sunheat chemicals Co., Ltd, Shanghai, China) were used for positive controls. Acetic acid was purchased from China National Pharmaceutical Group Corporation (Beijing, China). Formalin was obtained from Shanghai Lianshi Chemical Reagent (Shanghai, China). Complete Freund's adjuvant, allopregnanolone (3α,5α-tetrahydroprogesterone, 3α,5α-THP) and pregnenolone were purchased from Sigma-Aldrich (St. Louis, MO, USA), and methyltestosterone was purchased from National Institutes for Food and Drug Control (Beijing, China). 2-Nitro-4-trifluoromethylphenylhydrazine was synthesized in our laboratory as previously described (Higashi et al., 2002), with a purity of 98%. Methanol and Methyl cyanides were chromatographic pure and were obtained from Merck KGaA (Darmstadt, Germany). Other agents were analytical reagents.

Koumine, morphine, aspirin, indomethacin and gabapentin were dissolved or diluted in sterile physiological saline (0.9% NaCl) before use and were administered subcutaneously (s.c.) at a dose volume of 10 ml·kg^{-1} mouse body weight, 4 ml·kg^{-1} rat body weight. Acetic acid and formalin were diluted in sterile saline immediately before use.

2.2. In vivo acute toxicity determination

A total of 60 male ICR mice were randomly assigned to six groups of 10 animals. Koumine was administered s.c. at single doses of 66.6, 78.3, 92.1, 108.4, 127.5 and 150.0 mg·kg^{-1} respectively. Mice were observed for 2 weeks in all groups, and the mortality in each group was recorded. The median lethal dose (LD_{50}) was calculated using the Bliss method (Li et al., 1995).

2.3. Mouse acetic acid-induced inflammatory pain

To preliminary study the effect of koumine on pain, the acetic acid-induced inflammatory pain model (Koster et al., 1959) was conducted with minor modifications. Adult male ICR mice (n = 10 per group) were injected intraperitoneally with 10 ml·kg^{-1} of 0.6% acetic acid, and then the number of writhes was counted for 15 min. A writhe was defined as a contraction of the abdominal muscles accompanied by an elongation of the body and extension of the hindlimbs. Koumine (0.4, 2, 10 mg·kg^{-1}), aspirin (100 mg·kg^{-1}) or vehicle was administered s.c. 30 min prior to the acetic acid injection. The inhibitory rate and the effective rate of writhes were calculated using the following formula, respectively.% Inhibition = [(control responses − test responses)/control responses] × 100 (Hiramatsu et

al., 2001). When the inhibitory rate was 50% or more than that of the vehicle control group, the dose was considered effective. % Efficiency = number of mice in which the dose was effective / number of mice tested. The median effective dose (ED_{50}) was calculated from the effective rates according to the method of Litchfield and Wilcoxon (1949).

2.4. Mouse formalin-induced inflammatory pain

The formalin test was carried out according to Dubuisson and Dennis (1977), with minor modifications. Adult male ICR mice (n = 10 per group) received 10 μl of 5% formalin into the dorsal surface of the right hindpaw. Formalin induces biphasic pain behavior responses, divided into the phase I (0–5 min) and after interphase period with no pain behaviors, a phase II (11–60 min). The time spent licking or biting the affected hindpaw was recorded as nociceptive responses in 5-min bins for 1 h. Koumine (0.4, 2, 10 $mg \cdot kg^{-1}$), morphine (10 $mg \cdot kg^{-1}$), aspirin (100 $mg \cdot kg^{-1}$) or vehicle was administered s.c. 30 min prior to the formalin injection. The inhibitory rate of licking/biting time was calculated as the following formula for each phase, respectively. % Inhibition = [(control licking time − test licking time) / control licking time] × 100 (McGaraughty et al., 2003). The effective rate was calculated as the same as writhing test.

2.5. Mouse complete Freund's adjuvant (CFA) -induced inflammatory pain

Chronic inflammatory pain was induced according to Munro et al. (2008), with minor modifications. Adult male ICR mice received a subcutaneous injection of CFA (50% in saline, 20 μl) into the plantar surface of the right hindpaw. After the CFA injection, the animals were returned to their cages and continued to be handled daily for 7 days for habituation purposes. Koumine (0.8, 4, 20 $mg \cdot kg^{-1}$), indomethacin (10 $mg \cdot kg^{-1}$), or vehicle was administered orally once per day for 10 consecutive days beginning from day 9 after CFA injection. The thermal threshold was measured before CFA injection (baseline), day 8 (pre-dosing) after CFA injection, and 30 min after drug administration (post-dosing) of days 10, 12, 14, 16 and 18 after CFA injection. Thermal nociceptive thresholds were assessed using the procedure described below. Only mice with thermal baseline threshold score (calculated by CFA ipsilateral paw baseline/contralateral paw baseline) between 0.8 and 1.2 were used in the study. Moreover, after CFA treatment, mice with pre-dosing thermal threshold score at >0.8 were excluded. The inhibitory rate of koumine on thermal hyperalgesia was calculated as the following formula. % Inhibition = [(post-dosing threshold) − (pre-dosing threshold)] / [(baseline threshold) − (pre-dosing threshold)] × 100 (Walker et al., 2003). The effective rate was calculated as the same as writhing test.

2.6. Rat chronic constriction injury (CCI) model of neuropathic pain

The rat CCI model of neuropathic pain was produced according to the method described by Bennett and Xie (1988). Adult male Sprague–Dawley rats were anesthetized with chloral hydrate (400 $mg \cdot kg^{-1}$; intraperitoneally; Sigma-Aldrich). The right common sciatic nerve was isolated at mid-thigh level, and loosely ligated using four chromic gut (5–0) ties separated by an interval of 1 mm. For sham surgeries, the right sciatic nerve was exposed using the same procedure, but the nerve was not ligated. All animals were allowed 3 days to recover from the surgery. Koumine (0.28, 1.4, 7 $mg \cdot kg^{-1}$), gabapentin (40 $mg \cdot kg^{-1}$), or vehicle was administered s.c. twice per day for 7 consecutive days beginning from postoperative day 4. The thermal threshold was measured before surgery (baseline), drug treatment (pre-dosing), and 30 min after drug administration (post-dosing) on the morning of postoperative days 6, 8 and 10. Thermal and mechanical testing was measured using the procedures described below. Only rats with thermal and mechanical baseline threshold scores (calculated by CCI ipsilateral paw baseline/contralateral paw baseline) between 0.8 and 1.2 were used in the study. Moreover, after the surgery, rats with pre-dosing thermal threshold score at >0.8 or pre-dosing mechanical threshold scores >0.75 were excluded, and animals demonstrating motor deficit were excluded. The inhibitory rate of koumine on neuropathic thermal hyperalgesia and mechanical allodynia was calculated as the following formula. % Inhibition = [(post-dosing threshold) − (pre-dosing threshold)] / [(baseline threshold) − (pre-dosing threshold)] × 100 (Negus et al., 2006). The effective rate was calculated as the same as writhing test.

2.7. Rat L5 spinal nerve ligation (SNL) model of neuropathic pain

The rat L5 SNL model of neuropathic pain was conducted according to the method previously described by Kim and Chung (1992). Adult male Sprague–Dawley rats were placed under chloral hydrate anesthesia (400 $mg \cdot kg^{-1}$, intraperitoneally, Sigma-Aldrich) and a 1.5 cm incision was made dorsal to the lumbosacral plexus. The paraspinal muscles (right side) were separated from the spinous processes. Then, the L5 spinal nerves were isolated and tightly ligated with 3–0 silk suture distal to the dorsal root ganglion. Care was taken not to pull the nerve or contact the intact L4 Spinal nerve. In sham surgeries, the right L5 spinal nerve was exposed using the same procedure, but the nerve was not ligated. Following spinal nerve ligation, rats were given 3 days of recovery before behavioral testing ensued. Koumine (0.28, 1.4, 7 $mg \cdot kg^{-1}$), gabapentin (40 $mg \cdot kg^{-1}$), or vehicle was administered s.c. twice per day for 7 consecutive days from postoperative day 4. Thermal threshold was measured before surgery (baseline), drug treatment (pre-dosing), and 30 min after drug administration (post-dosing) on the morning of postoperative days 6, 8 and 10. Mechanical threshold was measured 30 min after thermal threshold measurement for each rat. Thermal and mechanical testing was measured according to the procedures described below. Only rats with thermal and mechanical threshold scores between 0.8 and 1.2 were used in the pharmacological experiments. After the surgery, rats with pre-dosing thermal threshold scores >0.6 or pre-dosing mechanical threshold scores >0.5 were excluded. The inhibitory rate and the effective rate of koumine on neuropathic thermal hyperalgesia and mechanical allodynia were calculated using the same procedures as CCI.

2.8. Measurement of thermal hyperalgesia

Thermal hyperalgesia was determined using a commercially available thermal paw stimulator (PL-200, Chengdu Technology & Market Co, Ltd, Sichuan, China) as described by Hargreaves et al. (1988). Rats were placed into individual plastic cubicles, which were mounted on a glass surface. A thermal stimulus, in the form of radiant heat emitted from a focused projection bulb, was then applied to the plantar surface of each hind paw. The maximum time of exposure was set at 16 s to limit possible tissue damage. Both hind paws of each rat were tested in two sequential trials at approximately 10 min intervals. Paw thermal withdrawal latency (TWL) was calculated as the mean of the two latencies.

2.9. Measurement of mechanical allodynia

Mechanical allodynia was measured using a commercially available electronic von Frey apparatus (Model 2390; IITC Life Science Inc., Woodland Hills, CA) as described by Mitrirattanakul et al. (2006) with minor modifications. Rats were placed into a Plexiglas box on a steel mesh floor and analyses were performed using an electronic von Frey apparatus. Stimulation was applied to the center of the hind paw in an upward motion of the von Frey filament until the foot withdrawal occurred, and the withdrawal threshold was

automatically recorded. The maximum strength of the filament used for von Frey testing was 55 g. The procedure was repeated twice at approximately 10 min intervals for each hind paw and mechanical withdrawal latency (MWL) was calculated as the mean of the two latencies.

2.10. Determination of neurosteroid levels by liquid chromatography–tandem mass spectrometry (LC–MS)

To investigate the possible mechanism by which koumine ameliorates neuropathic pain, adult male Sprague–Dawley rats with CCI or sham surgery were assigned into koumine treated groups, a vehicle control group, and a sham control group. Koumine (0.28, 1.4, 7 $mg \cdot kg^{-1}$), or vehicle was administered s.c. twice per day for 7 consecutive days beginning on postoperative day 4. Rats were killed by decapitation on postoperative day 11, and the lumber segments (L5-6) of the spinal cord were dissected, weighed and stored at −80 °C. The allopregnanolone and pregnenolone analyses were performed by a highly sensitive and specific High Performance Liquid Chromatography–Mass Spectrometry (HPLC–MS) method preceded by solid phase extraction purification, as previously described (Liere et al., 2000; Porcu et al., 2009) with minor modifications. Briefly, 100 mg samples of lumbar spinal cord were added with an internal standard (methyltestosterone) and homogenized in 0.5 ml of phosphate buffer using a glass homogenizer. The homogenate was then extracted by 1.5 ml of ethylacetate three times. The organic phases were combined and dried with a gentle stream of nitrogen in a 55 °C water bath. The samples were re-suspended with 1.5 ml of MeOH/H_2O (50:50, v/v) and filtered through a SPE cartridge, previously activated with MeOH (3 ml) and H_2O (5 ml). One ml of 80% MeOH/H_2O (80:20, v/v) eluted the SPE cartridge and the eluent was discarded. Finally, steroids were eluted in 2 ml of MeOH and dried again with a gentle stream of nitrogen in a 55 °C water bath. The dry residue was re-suspended in 2 ml of ethyl acetate/methanol (80:20, v/v) and the sample was filtered through a NH_2 column (Supelclean LC-NH_2, 500 mg, Supelco, Bellefonte, PA, USA) previously preconditioned with 3 ml of ethyl acetate and 3 ml of ethyl acetate/methanol (80:20, v/v). The neuroactive steroids passed unretained through the sorbent, and the eluate was collected. The NH_2 column was further rinsed with 2 ml more of the solvent mixture and the combined eluates in the derivatization tube were evaporated with a gentle stream of nitrogen in a 55 °C water bath before derivatization. Purification with the NH_2 column was necessary to improve the validation and accuracy of the assay.

Purified steroids were derivatized according to Higashi et al. (2002). After purification, the dried samples were dissolved in 100 µl of dehydrated alcohol and derivatized with 200 µl 2-nitro-4-trifluoromethylphenylhydrazine (0.10 $mg \cdot ml^{-1}$), followed by vortex mixing. Samples were allowed to react for 1 h at 65 °C in a water bath and were dried under a gentle stream of nitrogen. Derivatized samples were re-suspended in 100 µl of acetonitrile/H_2O (84:16, v/v) and 10 µl of each sample was injected into the HPLC–MS. The recoveries of the procedure were 88% and 86% for allopregnanolone and pregnenolone, respectively. The limits of allopregnanolone and pregnenolone detection with this method were 4 and 2 pg, respectively.

HPLC–MS analysis was carried out on an Agilent 1100 HPLC–MS (Agilent Technologies, Inc., Santa Clara, CA, USA). A column SB-C18, (2.1 mm × 75 mm ID, 3.5 µm, USA, Agilent Technologies, Inc.) equilibrated with 90% methanol was used to separate the derivatives of each neuroactive steroid at a flow rate of 0.5 $ml \cdot min^{-1}$. Samples (10 µl) were injected into the HPLC. The mass spectrometer was operated in the negative ion mode with the atmospheric-pressure chemical ionization source using nitrogen as a sheath, and auxiliary and sweep gas flow rates were 5 $L \cdot min^{-1}$. Other ion-source parameters: vaporizer temperature 250 °C, drying gas temperature 300 °C, ion-source collision-energy 180 V, capillary pressure 3.5 kV, allopregnanolone and pregnenolone were analyzed by multiple reaction monitoring. The secondary ion was m/z 175.

2.11. Statistical analysis

Unless otherwise stated, data indicate the mean ± S.E.M.. Data from the writhing and formalin tests, and neurosteroid determination, were analyzed using a one-way ANOVA with either a Dunnett T3 or a Bonferroni test for *post hoc* comparisons. Thermal and mechanical threshold response data were analyzed using a two-way ANOVA (treatment × time), followed by either a Dunnett T3 or a Bonferroni test for *post hoc* comparisons. *P* values less than 0.05 were considered statistically significant. All analyses were performed with SPSS (version 10.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Acute toxicity

The LD_{50} of koumine given subcutaneously to mice was 99.0 $mg \cdot kg^{-1}$, and the 95% confidence interval was 89.9–109.0 $mg \cdot kg^{-1}$. Acute lethality generally occurred within 10 min of subcutaneous injection; respiration became labored, and brief coordinated clonic convulsions occurred immediately before death.

3.2. Effects of koumine on acetic acid-induced inflammatory pain

As shown in Fig. 2, aspirin significantly inhibited the writhing responses induced by inflammatory pain, with an inhibitory rate of 78.1 ± 3.1% and an effective rate of 100%. Koumine produced a dose-

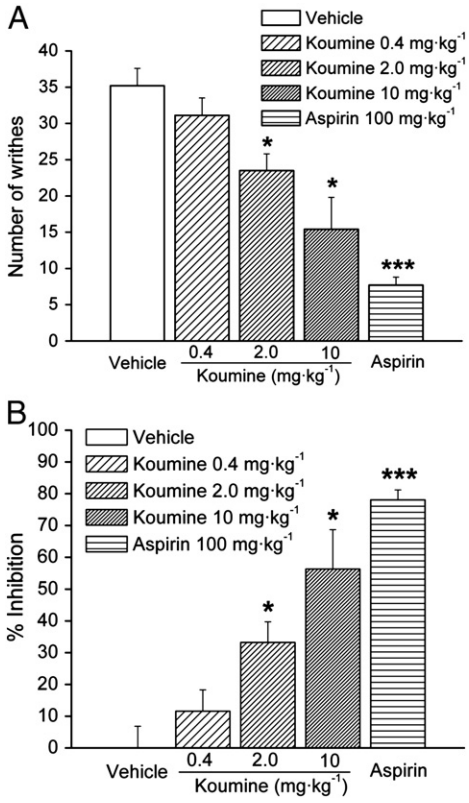

Fig. 2. Antinociceptive effects of koumine on acetic acid-induced inflammatory pain expressed as the number of writhes (A) and percent inhibition (B). Koumine (0.4, 2.0, 10 $mg \cdot kg^{-1}$), aspirin (100 $mg \cdot kg^{-1}$) or vehicle was administered s.c. 30 min prior to intraperitoneal injection of 0.6% acetic acid. Number of writhes was counted for 15 min. Data are expressed as mean ± S.E.M. (n = 10 per group). $^{*}P<0.05$, $^{***}P<0.001$ versus vehicle control animals, one-way ANOVA followed by Dunnett T3 test.

dependent decrease in the writhing counts ($F_{2,\ 27}=6.197$; $P<0.01$). The inhibitory rates of koumine were 11.6±6.7%, 33.2±6.5% ($P<0.05$ relative to vehicle), and 56.3±12.4% ($P<0.05$ relative to vehicle) at the subcutaneous doses of 0.4, 2, 10 mg·kg^{-1}, respectively. The effective rates of koumine at subcutaneous doses of 0.4, 2, 10 mg·kg^{-1} were 0%, 30% and 60%, respectively. The anti-writhing ED_{50} of koumine, calculated from the effective rates, was 5.85 mg·kg^{-1}. Although the writhing response of 10 mg·kg^{-1} koumine (15.4±4.4) was more than that of 100 mg·kg^{-1} aspirin (7.7±1.1), no significant difference was revealed ($P=0.725$). These data indicate that koumine likely possess the ability to ameliorate inflammatory pain.

3.3. Effects of koumine on formalin-induced inflammatory pain

The effect of koumine on formalin-induced nociceptive behaviors is shown in Fig. 3. Vehicle control rats produced overt behavioral signs in response to formalin, showing the first high peak within 5 min with a second phase of licking/biting that began 11 min. Koumine and aspirin did not significantly reduce Phase I nociceptive behaviors compared to vehicle ($P>0.05$), while morphine completely inhibited licking/biting during Phase I ($P<0.05$). In contrast, koumine dose-dependently reduced formalin-induced nociceptive behaviors in Phase II ($F_{2,\ 27}=14.467$, $P<0.001$); with doses of 2 and 10 mg·kg^{-1} producing a significant reduction in nociceptive behaviors ($P<0.001$ respectively, versus vehicle). Aspirin and morphine also significantly blocked Phase II ($P<0.05$ and $P<0.001$ respectively, versus vehicle) with an inhibitory rate of 47.4±10.1% and 96.6±1.9%, respectively and an effective rate of 60% and 100%, respectively. No significant difference was found when either 2 mg·kg^{-1} of koumine was administered (% inhibition: 58.2±4.0%) or 100 mg·kg^{-1} of aspirin was given ($P=0.991$). These data suggest that koumine is capable of relieving inflammatory pain.

3.4. Effects of koumine on CFA model of chronic inflammatory pain

Effects of koumine on chronic inflammatory pain were examined using a CFA model. A two-way ANOVA on TWL in the hindpaw ipsilateral to CCI demonstrated a significant treatment effect between subjects ($F_{5,\ 539}=665.1$, $P<0.001$) and time ($F_{6,\ 539}=359.8$, $P<0.001$) and a significant interaction between treatment and time ($F_{30,\ 539}=30.1$, $P<0.001$). *Post hoc* Dunnett T3 tests showed CFA treatment significantly decreased TWT to thermal stimulation ($P<0.001$, vs. naive control), demonstrating the development of thermal hyperalgesia from day 8 after CFA treatment to the end of the observation period. Indomethacin significantly reduced thermal hyperalgesia ($P<0.001$ vs. vehicle control). Administration of koumine (4, 20 mg·kg^{-1}, once per day beginning from day 9 to day 18 after CFA treatment) also reversed thermal hyperalgesia ($P<0.001$). At a dose of 20 mg·kg^{-1}, koumine produced more potent effects on thermal hyperalgesia ($P<0.001$) relative to indomethacin (10 mg·kg^{-1}). The analgesic effect of koumine was dose-dependent (two-way ANOVA, $F_{2,\ 185}=179.7$, $P<0.001$) and time-dependent (two-way ANOVA, $F_{4,\ 185}=23.3$, $P<0.001$). As shown in Fig. 4, repeated administration of koumine (20 mg·kg^{-1}) produced analgesic activity within 1 day after the first administration and reached the maximum protective effect on day 10 of treatment (inhibition: 65.9±4.9%; efficiency: 76.9%). These data suggest that koumine might reverse chronic inflammatory pain.

3.5. Effects of koumine on CCI model of neuropathic pain

The ability of koumine to reduce nociception in the formalin test prompted us to investigate the impact of koumine on regulating neuropathic pain. In an initial test, we measured the effects of koumine on the CCI model (Bennett and Xie, 1988). In a preliminary set of experiments, we examined the potential effects of koumine in sham CCI rats (Fig. 5A–B). A two-way ANOVA on TWL and MWL in the hindpaw ipsilateral to sham CCI operation demonstrated no significant treatment effect between subjects ($F_{1,\ 70}=2.035$, $P=0.158$ for TWL and $F_{1,\ 70}=1.075$, $P=0.303$ for MWL) and time ($F_{4,\ 70}=0.662$, $P=0.621$ for TWL and $F_{4,\ 70}=0.489$, $P=0.744$ for MWL) and no significant interaction between treatment and time ($F_{4,\ 70}=0.235$, $P=0.918$ for TWL and $F_{4,\ 70}=1.911$, $P=0.118$ for MWL). In the next step, we examined the effects of koumine in CCI rats (Fig. 5C–F). A two-way ANOVA on TWL and MWL in the hindpaw ipsilateral to CCI demonstrated a significant treatment effect between subjects ($F_{5,\ 205}=1883.7$, $P<0.001$ for

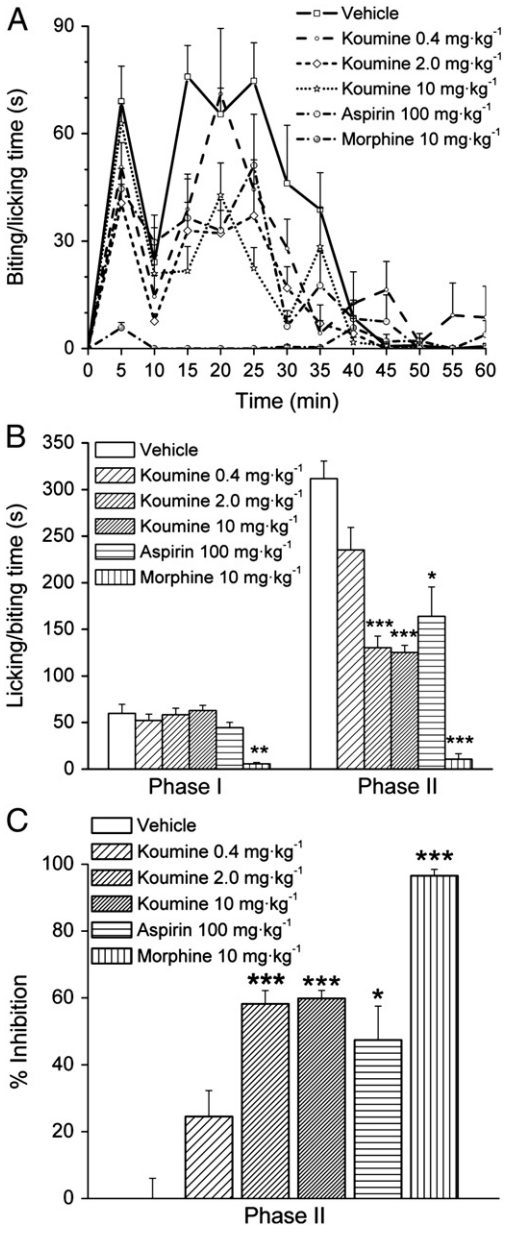

Fig. 3. Effects of subcutaneous administration of koumine on formalin-induced nociceptive behaviors in mice. Koumine (0.4, 2, 10 mg·kg^{-1}), aspirin (100 mg·kg^{-1}), morphine (10 mg·kg^{-1}) or vehicle was administered s.c. 30 min prior to subcutaneous injection of 5% formalin (10 μl) into the dorsal surface of the right hind paw. The formalin nociceptive behavior was measured for 1 h. The time course of hindpaw licking in 5 min bins (A), the cumulative time spent licking/biting in phase I (0–5 min) and phase II (11–60 min) (B), and % inhibition of nociceptive behavior (C). It is clear that subcutaneous vehicle-injected animals displayed typical biphasic behaviors, but that subcutaneous administration of koumine selectively and dose-dependently reduced the amount of nociceptive behaviors during the second phase. Data indicate mean± S.E.M. (n=10 per group). $^{*}P<0.05$, $^{***}P<0.001$ versus vehicle control group, one-way ANOVA followed by Dunnett T3 test.

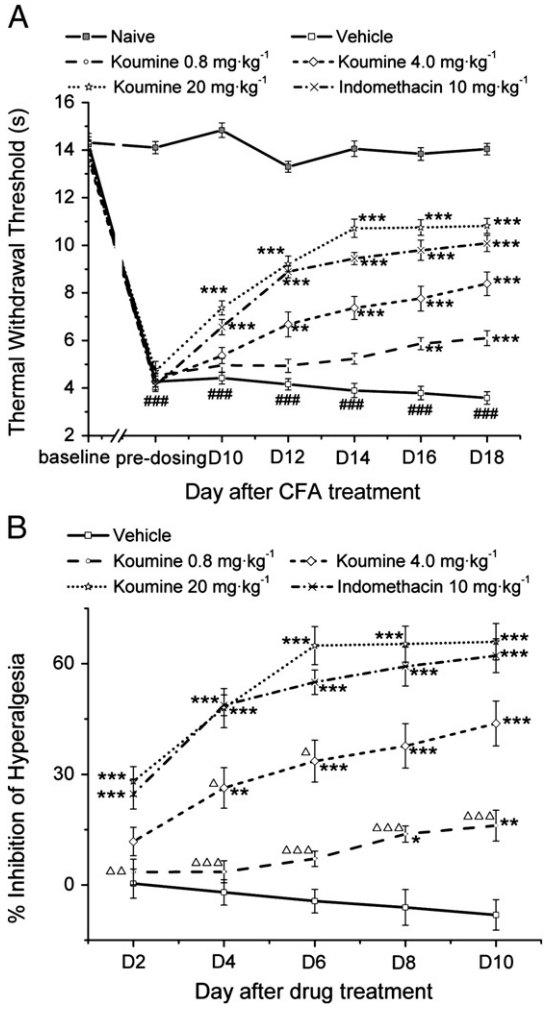

Fig. 4. Effects of repeated oral administration of koumine on CFA-induced thermal hyperalgesia in mice. Koumine (0.8, 4, 20 mg·kg^{-1}), indomethacin (10 mg·kg^{-1}), or vehicle was administered orally once per day for 10 consecutive days beginning from day 9 after CFA injection. The thermal threshold was measured before CFA injection (baseline), day 8 (pre-dosing) after CFA injection, and 30 min after drug administration (post-dosing) of days 10, 12, 14, 16 and 18 after CFA injection. The time course of koumine on thermal (A) withdrawal latency and % inhibition of thermal hyperalgesia (B) show that repeated oral injections of koumine dose-dependently reversed CFA-induced hyperalgesia. Data indicate withdrawal threshold for the ipsilateral paw as mean ± S.E.M. (n = 13–14 per group). Responses of the contralateral hindpaw remained unchanged throughout the procedure. $^{###}P<0.001$ versus sham group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus vehicle control group, $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, $^{\triangle\triangle\triangle}P<0.001$ versus indomethacin group, separate one-way ANOVA followed by Bonferroni or Dunnett T3 test for each time point.

TWL and $F_{5,\ 205} = 340.7$, $P<0.001$ for MWL) and time ($F_{4,\ 205} = 1911.7$, $P<0.001$ for TWL and $F_{4,\ 205} = 407.5$, $P<0.001$ for MWL) and a significant interaction between treatment and time ($F_{20,\ 205} = 203.6$, $P<0.001$ for TWL and $F_{20,\ 205} = 34.5$, $P<0.001$ for MWL). *Post hoc* Dunnett T3 tests showed CCI significantly decreased TWT to thermal stimulation ($P<0.001$ versus sham control) and MWT to mechanical stimulation ($P<0.001$ versus sham control), demonstrating the development of thermal hyperalgesia and mechanical allodynia, which peaked at post-operative days 8 and 10,respectively, and persisted for the entire observation period. Gabapentin significantly reduced thermal hyperalgesia ($P<0.001$) and mechanical allodynia ($P<0.05$) relative to vehicle. Administration of koumine (7 mg·kg^{-1}, twice per day beginning from post-operative day 4 to day 10) also significantly reversed thermal hyperalgesia ($P<0.001$) and mechanical allodynia ($P<0.01$) compared to vehicle. On the other hand, gabapentin (40 mg·kg^{-1}) did not show a statistical difference with 7 mg·kg^{-1} of koumine on thermal hyperalgesia ($P = 0.573$) and on mechanical allodynia ($P = 0.981$). The analgesic effect of koumine was dose-dependent (two-way ANOVA, $F_{2,\ 63} = 2019.5$, $P<0.001$ for TWL and $F_{2,\ 63} = 151.6$, $P<0.001$ for MWL) and time-dependent (two-way ANOVA, $F_{2,\ 63} = 164.4$, $P<0.001$ for TWL and $F_{2,\ 63} = 25.5$, $P<0.001$ for MWL). As shown in Fig. 5C–F, repeated administration of koumine (7 mg·kg^{-1}) exerted analgesic activity within 2 days after the first administration and reached the maximum protective effect on day 7 of treatment (% inhibition: 86.2 ± 2.0% for TWL and 81.7 ± 2.3% for MWL; % efficiency: 100% for both TWL and MWL). These data suggest that koumine might reverse neuropathic pain.

3.6. Effects of koumine on L5 SNL model of neuropathic pain

To further evaluate the impact of koumine on mitigating neuropathic pain, we measured the effects of koumine using the L5 SNL model (Kim and Chung, 1992). A two-way ANOVA on TWL and MWL in the hindpaw ipsilateral to L5 SNL demonstrated a significant treatment effect between subjects ($F_{5,\ 210} = 4778.0$, $P<0.001$ for TWL and $F_{5,\ 210} = 1046.9$, $P<0.001$ for MWL) and time ($F_{4,\ 210} = 9290.6$, $P<0.001$ for TWL and $F_{4,\ 210} = 1910.2$, $P<0.001$ for MWL) and a significant interaction between treatment and time ($F_{20,\ 210} = 616.2$, $P<0.001$ for TWL and $F_{20,\ 210} = 118.2$, $P<0.001$ for MWL). *Post hoc* Dunnett T3 test for TWL and Bonferroni test for MWL showed that L5 SNL significantly decreased both TWT ($P<0.001$) and MWT ($P<0.001$) relative to sham, demonstrating the development of thermal hyperalgesia and mechanical allodynia that peaked at post-operative day 4 and persisted throughout the observation period. Gabapentin significantly reduced both thermal hyperalgesia ($P<0.05$) and mechanical allodynia ($P<0.001$) compared to vehicle. Administration of koumine (7 mg·kg^{-1}, twice per day from post-operative day 4 to day 10) also significantly reversed thermal hyperalgesia ($P<0.01$). Moreover, koumine (0.28–7 mg·kg^{-1}) significantly reversed mechanical allodynia ($P<0.001$, versus vehicle respectively). At a dose of 7 mg·kg^{-1}, koumine produced more potent effects on mechanical allodynia ($P<0.001$) but not thermal hyperalgesia ($P = 0.995$) relative to gabapentin (40 mg·kg^{-1}). The analgesic effect of koumine was dose-dependent (two-way ANOVA, $F_{2,\ 63} = 2969.6$, $P<0.001$ for TWL and $F_{2,\ 63} = 398.7$, $P<0.001$ for MWL) and time-dependent (two-way ANOVA, $F_{2,\ 63} = 1307.7$, $P<0.001$ for TWL and $F_{2,\ 63} = 232.1$, $P<0.001$ for MWL). As shown in Fig. 6, repeated administration of koumine (7 mg·kg^{-1}) exerted analgesic activity within 2 days after the first administration and reached the maximum protective effect on day 7 of treatment (% inhibition: 92.5 ± 1.2% for TWL and 81.7 ± 2.8% for MWL; % efficiency: 100% for both TWL and MWL). These data suggest that koumine mitigates neuropathic pain.

3.7. Effects of koumine on neurosteroids in rat lumbar spinal cord with CCI neuropathy

As shown in Fig. 7, allopregnanolone and pregnenolone were significantly increased in the CCI rats ($P<0.05$) relative to sham rats. Koumine treated rats elevated the level of allopregnanolone in the L5-6 spinal cord in a dose-related manner ($F_{2,\ 17} = 12.3$, $P<0.001$), and reached statistical significance at 7 mg·kg^{-1} ($P<0.01$) compared to vehicle. However, koumine treated rats showed a mild reduction in the levels of pregnenolone in the L5-6 spinal cord ($P>0.05$) relative to vehicle treated rats. These data suggest that koumine alleviates the effects of CCI neuropathy by up-regulating allopregnanolone in the spinal cord.

4. Discussion

Here we report the novel finding that koumine, the main alkaloidal constituent of *G. elegans* Benth., significantly reduces both inflammatory and neuropathic pain in multiple preclinical models. To our

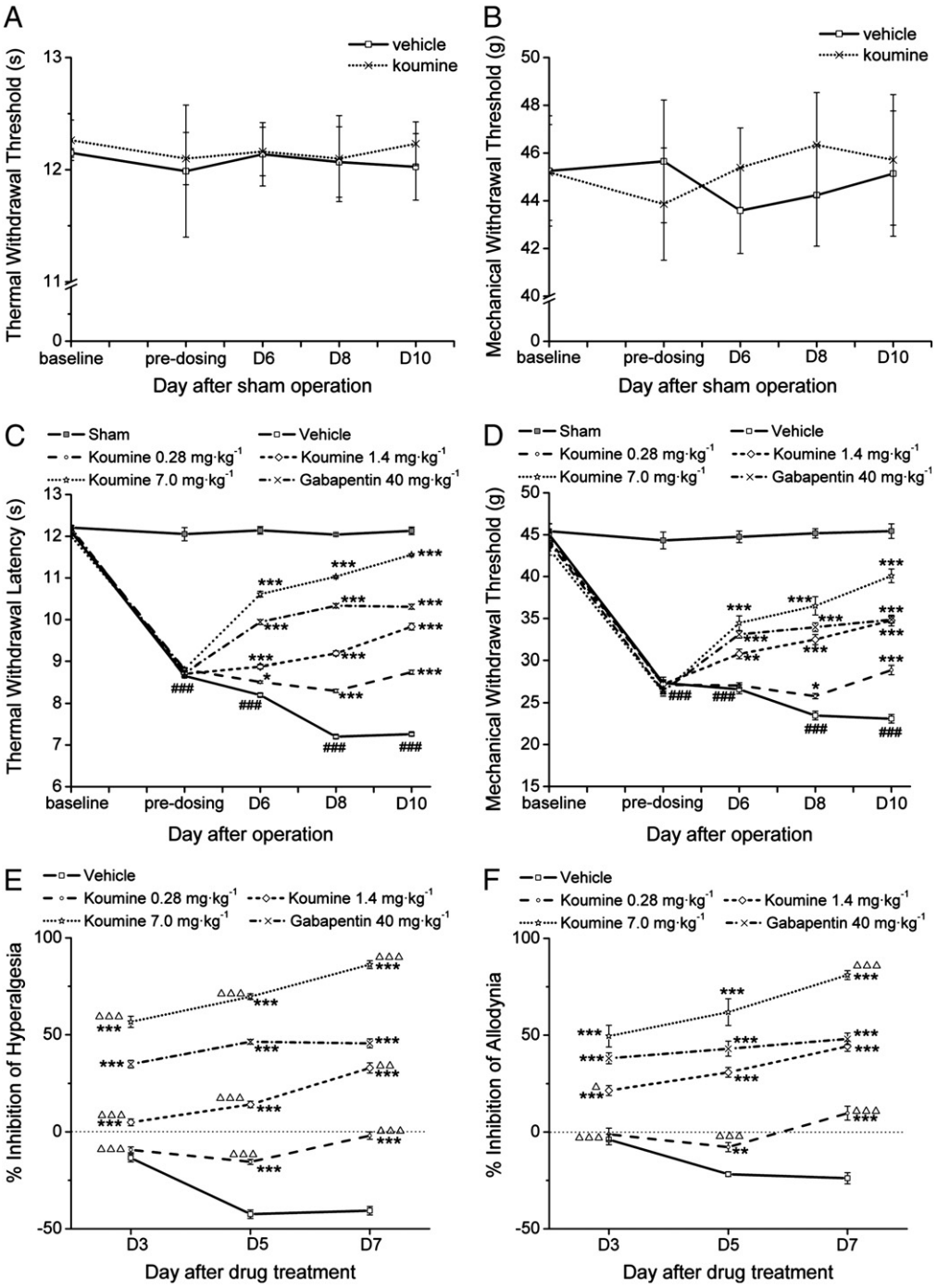

Fig. 5. Effects of repeated subcutaneous administration of koumine on thermal hyperalgesia and mechanical allodynia in rats with CCI neuropathy. Koumine (0.28, 1.4, 7 mg·kg^{-1}), gabapentin (40 mg·kg^{-1}), or vehicle was administered s.c. twice per day for 7 consecutive days from postoperative day 4. Thermal threshold was measured before surgery (baseline), drug treatment (pre-dosing), and 30 min after drug administration (post-dosing) on the morning of postoperative days 6, 8 and 10. The mechanical threshold was measured 30 min following the thermal threshold measurement for each rat. The time course of koumine on thermal (A) and mechanical (B) withdrawal latency in sham CCI rats indicate that repeated subcutaneous injections of koumine did not affect the threshold of sham CCI rats. The time course of koumine on thermal (C) and mechanical (D) withdrawal latency and % inhibition of thermal hyperalgesia (E) and mechanical allodynia (F) show that repeated subcutaneous injections of koumine dose-dependently reversed hyperalgesia and allodynia induced by CCI neuropathy. Data indicate withdrawal threshold for the ipsilateral paw as mean ± S.E.M. (n = 7–8 per group). Responses of the contralateral hindpaw remained unchanged throughout the procedure. $^{\#\#\#}P<0.001$ versus sham group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus vehicle control group, $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, $^{\triangle\triangle\triangle}P<0.001$ versus gabapentin group, separate one-way ANOVA followed by Bonferroni or Dunnett T3 test for each time point.

knowledge, this is the first evidence that koumine produces analgesic properties. Importantly, koumine may provide an alternative avenue of therapy for patients with intractable inflammatory and neuropathic pain.

This study was inspired by both clinical and preclinical findings showing that the crude alkaloidal extraction of *G. elegans* Benth. produces analgesic activity. For example, Chen (1984) reported that a parenteral solution of crude *Gelsemium* alkaloidal extraction is a potent analgesic for cancer pain and colicky pain with a rapid onset (within 15 to 30 min), and that the therapeutic effect was more efficacious than meperidine (Chen, 1984). Preclinical evidence (Tan et al., 1988) has demonstrated that the crude *Gelsemium* alkaloidal extraction exerted antinociceptive effects without tolerance and dependence relative to morphine. The crude *Gelsemium* alkaloidal

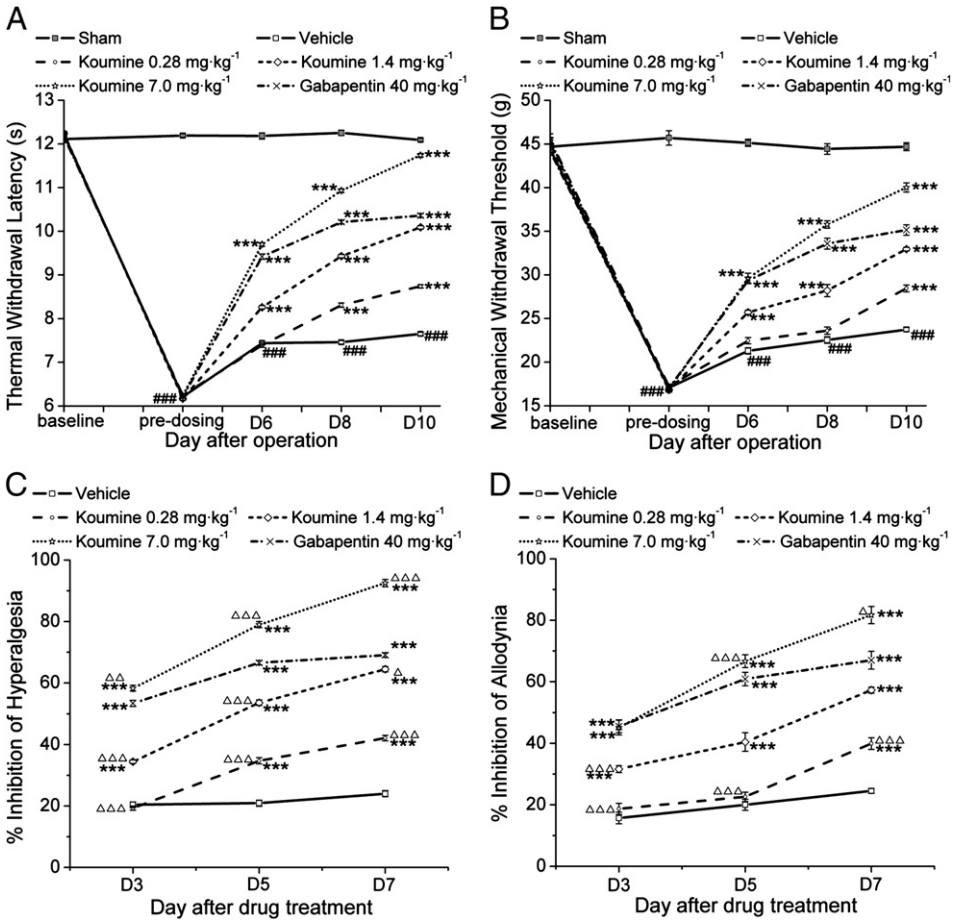

Fig. 6. Effects of repeated subcutaneous administration of koumine on thermal hyperalgesia and mechanical allodynia in rats with L5 SNL neuropathy. Koumine (0.28, 1.4, 7 mg·kg^{-1}), gabapentin (40 mg·kg^{-1}), or vehicle was administered s.c. twice per day for 7 consecutive days beginning from postoperative day 4. Thermal threshold was measured before surgery (baseline), drug treatment (pre-dosing), and 30 min after drug administration (post-dosing) on the morning of postoperative days 6, 8 and 10. Mechanical threshold was measured 30 min after the thermal threshold measurement for each rat. The time course of koumine on thermal (A) and mechanical (B) withdrawal latency and% inhibition of thermal hyperalgesia (C) and mechanical allodynia (D) indicate that repeated subcutaneous injections of koumine dose-dependently reversed the hyperalgesia and allodynia of L5 SNL neuropathy. Data show withdrawal threshold for the ipsilateral paw as mean ± S.E.M. (n = 8 per group). Responses of the contralateral hindpaw remained unchanged throughout the procedure. $^{\#\#\#}P<0.001$ versus sham group, $^{***}P<0.001$ versus vehicle control group, $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, $^{\triangle\triangle\triangle}P<0.001$ versus gabapentin group, separate one-way ANOVA followed by Bonferroni or Dunnett T3 test for each time point.

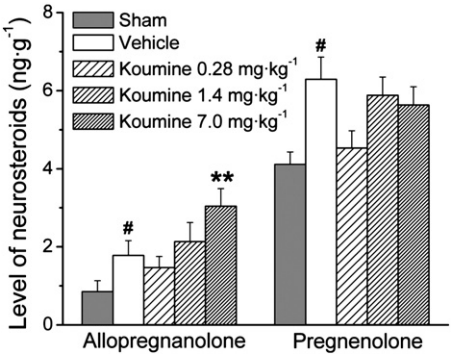

Fig. 7. Effects of repeated koumine on the levels of allopregnanolone and pregnenolone in rat lumbar spinal cord with CCI neuropathy. After CCI or sham surgery, koumine (0.28, 1.4, 7 mg·kg^{-1}), or vehicle was administered s.c. twice per day for 7 consecutive days beginning from postoperative day 4. On postoperative day 11, the lumbar segment (L5-6) of the spinal cord was dissected and the levels of allopregnanolone and pregnenolone were determined by high performance liquid chromatography–tandem mass spectrometry. Data are expressed as mean ± S.E.M. (n = 6 per group). $^{\#}P<0.05$ versus sham group, $^{**}P<0.01$ versus vehicle control group, one-way ANOVA followed by Bonferroni test.

extraction failed to show an obvious antipyretic effect (Tan et al., 1988). Therefore, *Gelsemium* alkaloids possess a potent analgesic action that is different than the underlying mechanisms of either opioids or NSAIDs. Unfortunately, the crude *Gelsemium* alkaloidal extraction has a high level of toxicity. For example, the LD_{50} was 1.56 (1.4–1.69) mg·kg^{-1} of intravenous injection in mice, and 1.2 (0.8–1.7) mg·kg^{-1} of intraperitoneal injection in rats (Tan et al., 1988). Thus, testing crude *Gelsemium* alkaloidal extraction in future clinical utility has been hampered. However, the unique analgesic mechanism, as well as, the nonuniformity of the monomer toxicity of *Gelsemium* alkaloids provoked a deep interest in its clinical viability. Therefore, we established a method to isolate alkaloid monomers from *Gelsemium* by pH-zone-refining counter-current chromatography (Su et al., 2011), which allowed abundant collection of highly purified *Gelsemium* alkaloid monomers. For instance, we obtained generous koumine with purity of 99%, enabling investigation of the pharmacology and toxicity of koumine.

First, we evaluated the acute toxicity of subcutaneous injections of koumine and found that it occurred within a couple of minutes after injection. The toxic signature was similar to that of the crude alkaloidal extraction (Rujjanawate et al., 2003). The calculated LD_{50} of subcutaneous injection of koumine was 99 mg·kg^{-1}, which was similar to that of the intraperitoneal injection route of koumine (100 mg·kg^{-1}) (Zhang, 1987).

To evaluate the effect of koumine on inflammatory pain, we used three standard models. First, the acetic acid-induced pain model, which is occasionally classified as a visceral pain model, is a very sensitive method to test novel molecules with unknown pharmacodynamic properties (Le Bars et al., 2001). We demonstrated that koumine dose-dependently inhibited the acetic acid-induced writhing responses with an ED_{50} of 5.85 $mg \cdot kg^{-1}$. According to the above determined LD_{50} and ED_{50}, the therapeutic index of subcutaneous koumine was approximately 17. According to Rujjanawate et al. (2003), the ED_{50} of intraperitoneal crude *Gelsemium* alkaloidal extraction in mice was approximately 1 $mg \cdot kg^{-1}$ and the LD_{50} was 4 $mg \cdot kg^{-1}$, thus the therapeutic index of intraperitoneal crude *Gelsemium* alkaloidal extraction was estimated to be approximately 4, which is far narrower than that of koumine. These data suggest that koumine may be a safe analgesic. Although, the writhing test has historically shown sensitivity to analgesics of both central and peripheral analgesia (Ferreira and Vane, 1974; Fukawa et al., 1980), the specificity is poor (Berge, 2011; Taber, 1973). Thus, the formalin-induced inflammatory pain model was executed to further validate the analgesic activity of koumine in inflammatory pain. In fact, the formalin test is undoubtedly one of the most predictive models of pain (Le Bars et al., 2001). Formalin produces a biphasic behavioral reaction resembling acute and tonic pain, respectively. Both phases of the behavioral response are associated with a primary afferent drive that would be expected to initiate and maintain activity-dependent sensitization at the spinal level (Berge, 2011). Central pain modulation occurs in the second phase of the test (Abbadie et al., 1997; Berge, 2011; Dickenson and Sullivan, 1987; McCall et al., 1996) and the release of inflammatory mediators is thought to be involved in the mechanism. Our results showed that a single dose of koumine produced a selective and profound inhibition of the second phase of the formalin test, suggesting that koumine possesses an analgesic action in response to tonic inflammatory pain by interfering with the release of inflammatory mediators during the central modulation response. CFA model produces more protracted hyperalgesia lasting for several days to weeks, which is used to model chronic inflammatory pain (Negus et al., 2006). Repeated oral koumine reversed CFA-induced long-lasting hyperalgesia, suggesting an efficacy against chronic inflammatory pain.

Vissers et al. (2006) reported that the second phase of the formalin test is correlated to the cold allodynia in the CCI model, which is one of the best characterized models of neuropathic pain. Thus, the significant antinociceptive effect of koumine on the second phase of the formalin prompted us to assume that koumine may have analgesic properties for neuropathic pain. A number of experimental models for neuropathic pain have been developed (Berge, 2011; Jaggi et al., 2011). The CCI and SNL models are two of the most extensively used animal models of painful peripheral mononeuropathy (Jaggi et al., 2011; Wang and Wang, 2003). These two models both successfully produce both long-lasting thermal hyperalgesia and mechanical allodynia, mimicking causalgia or complex regional pain syndrome in patients (Jaggi et al., 2011). Considering that drug therapies for neuropathic pain are dosed over days and weeks (Berge, 2011), long-term exposure of koumine was used, rather than a single dose. Repeated administration of koumine dose-dependently reversed thermal hyperalgesia and mechanical allodynia in both our CCI and SNL models, suggesting that koumine may be efficacious at least in peripheral neuropathic pain. Notably, LaBuda and Little (2005) have revealed that gabapentin, morphine and other agents completely, or partially, reverse tactile allodynia of L5 SNL animals, while indomethacin, a NSAID, is not efficacious. Thus, the positive analgesic effect of koumine on the L5 SNL model suggests that the analgesic action of koumine is different than that of NSAIDs. Moreover, koumine did not affect pain thresholds in the unstimulated contralateral hindlimb. Thus, the localized action of koumine to the area of tissue injury suggests that koumine functions only in afferents stimulated by noxious inputs.

Our behavioral data indicate that koumine ameliorates inflammatory and neuropathic pain. Results from experiments in sham CCI rats failed to reveal significant effects of koumine (7 $mg \cdot kg^{-1}$) on either TWL or MWL in sham CCI rats, suggesting that the observed effects in the CCI rats were not due to a change in the baseline. Furthermore, the analgesic actions are not due to hypomobility, as subcutaneous injections of the varying doses of koumine did not alter locomotor activity (data not shown). Moreover, repeated subcutaneous administration of koumine did not produce physical and psychological dependence (data not shown).

The pathophysiology and neurobiology of chronic pain are complex and not well understood. A growing body of evidence indicates that endogenous neurosteroids are involved in the modulation of chronic pain. The endogenous biosynthesis of neurosteroids, such as allopregnanolone and pregnenolone, is upregulated in the spinal cord during bouts of neuropathic pain (Mensah-Nyagan et al., 2008). Indeed, we observed a similar increase in both allopregnanolone and pregnenolone in CCI rats. The observed increase of allopregnanolone in CCI rats is consistent with that in SNL neuropathy reported by Kawano et al. (2011). Endogenous increased synthesis of 3α,5α-neurosteroids, such as allopregnanolone, in the spinal cord may represent an intrinsic adaptive response to neuropathic pain, inducing beneficial actions against diverse pathological symptoms (Kawano et al., 2011; Mensah-Nyagan et al., 2008; Meyer et al., 2008, 2010, 2011). However, inadequate increase of allopregnanolone failed to reverse the development of neuropathic pain, as seen in CCI rats. Only adequate increase of allopregnanolone could successfully protect animals against the development of neuropathic pain (Kawano et al., 2011). Our data revealed that koumine further increased allopregnanolone, but not pregnenolone in the spinal cord of CCI rats, suggesting that the anti-neuropathic pain activity of koumine is mediated by further upregulation of allopregnanolone to an adequate level against neuropathic pain. Potential mechanisms underlying such action could include T-type calcium blockade and $GABA_A$ receptor activation (Meyer et al., 2011; Mensah-Nyagan et al., 2008; Pathirathna et al., 2005). Interestingly, it has been reported that *G. sempervirens* and gelsemine could dose-dependently stimulate allopregnanolone synthesis through activation of glycine receptor, which may stimulate allopregnanolone-synthesizing enzymatic activity (Venard et al., 2011). Considering that koumine and gelsemine are structurally similar, we speculate that koumine might stimulate glycine receptor and then activate allopregnanolone synthesizing enzyme to increase allopregnanolone production.

Additionally, our preliminary findings suggest that gelsenicine, the most toxic alkaloid in *G. elegans*, exerts analgesic activity in the face of inflammatory and neuropathic pain (Liu et al., 2011). However, koumine is the most abundant alkaloid in *G. elegans*, which enables better isolation and acquisition compared to gelsenicine. Furthermore, the therapeutic index of koumine is wider than gelsenicine, suggesting that koumine will be more promising in clinical applications. By comparing the structures of gelsenicine and koumine we found that, other than the indole ring, their molecular formulas were quite different. Thus, we hypothesize that the indole ring may be the requisite for analgesia and that perhaps some specified stereo-conformations may be needed. It will be important for future studies to determine the structure–activity relationship between the *G. elegans* alkaloids.

We highlight that drug discovery from medicinal plants has been continuing to provide an important source of new drugs, new drug leads, and new chemical entities (Balunas and Kinghorn, 2005; Newman et al., 2000, 2003). A number of natural drugs derived from plants have been clinically effective for relieving different forms of pain. For example, some plant-derived analgesics include morphine, acetylsalicylic acid, and capsaicin. The important contribution of these compounds in developing new analgesics and their importance in understanding the complex pathways related to

electrophysiological and molecular mechanisms associated with pain physiology and pathophysiology should not be underestimated. It is clear that the prominent pain relief exhibited by koumine in this study points to yet another plant derivative as a possible analgesic. Of course, more research is needed to develop koumine into a safe and efficacious analgesic for inflammatory and neuropathic pain in human patients.

5. Conclusions

In summary, our results demonstrate that koumine has a significant analgesic action in rodent behavioral models of inflammatory and neuropathic pain, and that the reduction of neuropathic pain is associated with the modulation of neurosteroids in the spinal cord. While recognizing that animal models of inflammatory and neuropathic pain do not completely model clinical pain, our studies raise the possibility that koumine will prove useful in the treatment of a variety of intractable pain conditions in patients.

Acknowledgments

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