

Acupoint Stimulation With Diluted Bee Venom (Apipuncture) Potentiates the Analgesic Effect of Intrathecal Clonidine in the Rodent Formalin Test and in a Neuropathic Pain Model

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Abstract: Although intrathecal (i.t.) administration of the α_2 -adrenoceptor agonist clonidine has a pronounced analgesic effect, the clinical use of clonidine is limited by its side effects. Previously, our laboratory has demonstrated that the subcutaneous injection of diluted bee venom (DBV) into an acupoint (termed *apipuncture*) produces significant analgesic effect in various pain animal models. The present study was designed to examine whether DBV injection into the Zusanli acupoint (ST-36) could enhance lower-dose clonidine-induced analgesic effects without the development of hypotension, bradycardia, or sedation. In the mouse formalin test, DBV injection produced a dramatic leftward shift in the dose-response curve for clonidine-induced analgesia. In a rat neuropathic pain model i.t. clonidine dose dependently suppressed chronic constriction injury (CCI)-induced mechanical allodynia and thermal hyperalgesia, and this clonidine-induced analgesic effect was significantly potentiated by apipuncture pretreatment. DBV apipuncture alone or in combination with a low dose of i.t. clonidine produced an analgesic effect similar to that of the high dose of clonidine, but without significant side effects. The analgesic effect produced by the combination of i.t. clonidine and apipuncture was completely blocked by pretreatment with an α_2 -adrenoceptor antagonist. These data show that DBV-apipuncture significantly enhances clonidine-induced analgesia and suggest that a combination of low dose clonidine with acupuncture therapy represents a novel strategy for pain management that could eliminate clonidine's side effects.

Perspective: This study demonstrated that intrathecal clonidine-induced analgesia is significantly enhanced when it is combined with chemical acupuncture treatment. The administration of low-dose clonidine in combination with acupuncture produced a potent analgesic effect without significant side effects and thus represents a potential novel strategy for the management of chronic pain.

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Key words: Clonidine, diluted bee venom, neuropathic pain, formalin test, α_2 -adrenoceptor.

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Nerve injury produces neuropathic pain, which is associated with the development of a long-lasting hyperalgesia (increased response to noxious stimuli) and allodynia (a decrease in pain threshold).⁵¹ Neuropathic pain is often not relieved adequately by conventional pharmacological treatments including nonsteroidal antiinflammatory drugs and opioids.³¹ On the other hand, clonidine, a classic α_2 -adrenoceptor agonist, reverses both spontaneous and elicited pain in human neuropathic pain patients following spinal administration.⁴⁹ In support of clonidine's antinociceptive effect, neuropathic rats have been shown to self-administer intrathecal (i.t.) infusions of clonidine following sciatic nerve ligation in a stable and dose-responsive manner.³² Although clonidine is an effective analgesic in human patients, the high doses of clonidine typically required have been shown to cause serious side effects including disruption of motor coordination, hypotension and bradycardia.^{29,44} As a result of these side effects, the clinical use of clonidine is limited. Because of this, several groups have used a combination regime to treat chronic pain patients that involves the administration of a low dose of clonidine and an additional analgesic drug.^{27,28,41,42}

Subcutaneous injection of diluted bee venom (DBV) into an acupoint is a form of chemical acupuncture, termed *apipuncture*. Apipuncture has been used clinically in traditional Oriental medicine to produce a potent analgesia in chronic pain patients. Recent experimental studies in our laboratories provide support for this alternative medicine approach by demonstrating that subcutaneous injection of DBV (0.01 to 1 mg/kg) into the Zusanli acupuncture point (ST36) produces a prominent antinociceptive and antihyperalgesic effect in animal models of acute and persistent pain, respectively.^{17,22,23,37} We have further shown that this apipuncture-induced analgesic effect is mediated by the activation of descending bulbospinal noradrenergic pathways, which in turn activate spinal α_2 -adrenoceptors.^{19,22,24,25,38} Importantly we have also demonstrated that DBV-induced apipuncture does not produce any significant effects on general physiological functions including cardiovascular functions nor does it affect general motor activity even at the highest dose (1 mg/kg) tested.¹⁸ Based on these data, we hypothesized that a combined treatment strategy of i.t. clonidine in conjunction with apipuncture would have a synergic effect on spinal α_2 -adrenoceptors and thus produce a more prolonged and potentiated analgesic effect than that of either clonidine or apipuncture alone. In addition, we theorized that administration of a low dose of clonidine in combination with apipuncture would produce a significant antinociceptive/antihyperalgesic effect in rodent models of acute and chronic pain without producing the side effects that accompany higher doses of clonidine.

To test this hypothesis, we first evaluated whether the analgesic effect produced by i.t. clonidine was potentiated by pretreatment with apipuncture in the mouse acute formalin test and in the rat chronic constriction

injury (CCI)-induced neuropathic pain model. We then evaluated whether the combination of clonidine and apipuncture produced sedation, hypotension and bradycardia, the typical side effects of clonidine treatment by this combination. In the final experiment we pretreated the animals an α_2 -adrenoceptor antagonist in order to determine if the analgesic effect produced by the combined clonidine/apipuncture treatment is in fact mediated by activation of spinal α_2 -adrenoceptors.

Methods

Animals

Experiments were performed on male ICR mice (20 to 25 g) and Sprague-Dawley rats (250 to 300g). All experimental animals were obtained from the Laboratory Animal Center of Seoul National University in Korea. They were housed in colony cages with free access to food and water and maintained in temperature and light controlled rooms ($23 \pm 2^\circ\text{C}$, 12/12-hour light/dark cycle with lights on at 7 AM) for at least 1 week before the study. All of the methods used in the present study were approved by the Animal Care and Use Committee at Seoul National University and conform to NIH guidelines (NIH publication No. 86-23, revised 1985). All algiesometric assays were conducted under the ethical guidelines set forth by the International Association for the Study of Pain (IASP).

Intrathecal Drug Treatment

Clonidine or idazoxan (an α_2 -adrenoceptor antagonist, 10 $\mu\text{g}/\text{mouse}$, 50 $\mu\text{g}/\text{rat}$) was administered by i.t. injection, based on the technique developed by Hylden and Wilcox.¹⁴ Briefly, for mouse i.t. injections, a 30-gauge needle (length, 0.5 inch) connected to a 50- μL Hamilton syringe was inserted into the subarachnoid space between lumbar vertebrae L5 and L6. A flick of the mouse's tail provided a reliable indicator that the needle had penetrated the dura. The syringe was held in position for a few seconds after the injection of a volume of 5 μL /mouse. For rat i.t. injections, 10 μL of vehicle or drug was injected using a 26-gauge needle (length, 1 inch) connected to a 50- μL Hamilton syringe.⁹ The i.t. procedure for rats was similar to that of mice. Clonidine and idazoxan were purchased from Sigma (St Louis, MO) and diluted in saline.

Apipuncture With DBV

BV from *Apis mellifera* (Sigma) was dissolved in physiological saline. A BV dose of 0.01 mg/kg/50 μL was used for rats, whereas 0.25 mg/kg/20 μL of BV was administered to mice.^{37,38} DBV was subcutaneously injected into the right Zusanli acupoint (ST-36) located on the lateral side of the stifle joint adjacent to the anterior tubercle of the tibia as previously described.^{17,37,38} The anatomical location of the Zusanli acupoint in rats and mice is equivalent to that described in humans and the location of this acupoint was identified based on the description provided by Stux and Pomeranz⁴⁶ and on the location de-

picted in rat anatomical reference.⁴⁰ Animals in the control group received an injection of vehicle into the same site. In all apipuncture-clonidine experiments, DBV was injected 5 minutes before clonidine.

Formalin-Induced Pain Behaviors

The formalin test is an analgesic behavioral observation assessment method that has 2 phases of nociceptive behavior representing 2 different types of pain. Phase 1 is pain produced by direct nerve stimulation and phase 2 is an inflammation-induced pain.³⁵ In the present study mice were first acclimatized for 30 minutes in an acrylic observation chamber (30 cm in diameter and in height) and then 20 μ L of 1% formalin was injected subcutaneously into the plantar surface of the right hind paw with a 30 gauge needle as previously described.³⁸ Following formalin injection, the animals were immediately placed in a test chamber, and nociceptive responses in each animal were recorded using a video camera for a period of 30 min. The summation of time (in seconds) spent licking the formalin-injected hind paw during each 5-minute block was measured as an indicator of the nociceptive response. Two experienced investigators, who were blinded to the experimental conditions, measured the formalin-induced behaviors. The duration of the responses during the first 10-minute period represented the first phase, while the duration of responses during the subsequent 20-minute period (from 10 to 30 minutes after injection) represented the second phase of the formalin test. In this experiment clonidine was injected 5 minutes after DBV injection, but 5 minutes before formalin injection. Control groups consisted of mice that were injected with vehicle instead of clonidine and DBV before formalin injection.

Induction of Neuropathic Pain and Nociceptive Behavioral Assessment

A CCI of the sciatic nerve was performed according to the method described by Bennett and Xie.¹ Briefly, rats were anesthetized with 3% isoflurane in a mixture of N₂O/O₂ gas. The right sciatic nerve was exposed at the mid-thigh level, and 4 loose ligatures of 4-0 chromic gut were placed around the dissected nerve with a 1.0- to 1.5-mm interval between each ligature. Behavior assessments were performed 1 day before the neuropathic surgery to obtain normal values of withdrawal responses to heat and mechanical stimuli. The paw withdrawal latency (PWL) to thermal radiant heat stimuli is a widely used nociceptive measure to study hyperalgesic mechanisms. In the present study we assessed nociceptive responses to heat stimuli by measuring PWL based on the procedure previously described by Hargreaves et al.¹¹ Briefly, rats were placed in a plastic chamber (20 cm in diameter and length) with a glass floor and were allowed to acclimatize for 20 minutes before testing. A radiant heat source was positioned under the glass floor beneath the hind paw to be tested, and withdrawal latency was measured using plantar analgesia meter (IITC Life Science Inc, Woodland Hills, CA). The intensity of the

light source was calibrated to produce a withdrawal response within 10 to 12 seconds in normal animals. The test was duplicated in each hind paw, and the mean withdrawal latency was calculated. Cutoff time in the absence of a response was set at 20 seconds.

Mechanical allodynia testing was performed on rats that were first placed on a metal mesh grid under a plastic chamber as previously reported.^{37,39} A von Frey filament (North Coast Medical, Morgan Hill, CA) was then inserted through the grid and applied to the plantar surface of the hind paw. The number of paw withdrawal responses was measured to a normally innocuous von Frey filament of 2.0 g. Briefly, the von Frey filament was applied 10 times (once every 10 seconds) to each hind paw. The number of paw withdrawal responses after each stimulus was then counted. The frequency of foot withdrawal out of 10 trials with the von Frey filament application was expressed as a percentage of paw withdrawal response frequency (PWF, %). This was calculated as follows: the response rate (%) = number of foot withdrawals/number of trials \times 100. Fourteen days after CCI surgery, both thermal hyperalgesic and mechanical allodynic behaviors were assessed to confirm the induction of neuropathic pain.

Fos Immunohistochemistry and Image Analysis

Fos immunohistochemistry was performed on spinal cord tissue sections obtained 2 hours post-formalin injection. This time point was selected because spinal Fos protein expression typically reaches peak values at approximately 2 hours after acute peripheral stimulation as previously described.^{17,38} Mice were deeply anesthetized with 5% isoflurane and perfused by 0.2% picric acid in 0.1M phosphate buffer (pH 6.9). The spinal cords were removed immediately after perfusion, post-fixed in the identical fixative for 4 hours and then placed in 30% sucrose in phosphate-buffered saline (pH 7.4) overnight at 4°C. Serial transverse sections (40 μ m) of the spinal cord were cut using a cryostat (Microm, Germany). Spinal L3-L6 tissue sections were processed for Fos (rabbit polyclonal anti-Fos antibody; Calbiochem, EMD Biosciences, San Diego, CA; 1:10000) immunohistochemistry using the avidin-biotin-peroxidase procedure as previously described.¹⁷ Fos-like immunoreactive (FLI) neurons were visualized using a 3-3 diamino-benzidine (Sigma) reaction. The mean number of FLI neurons per spinal cord region per group was determined by averaging the number of FLI cells in 5 spinal cord sections (containing the greatest number of FLI neurons from the L3-L6 cord segments) from each animal in the group. All Fos quantitative analysis were performed as previously described using a cooled CCD (Micromax Kodak 1317; Princeton Instrument, Tucson, AZ) equipped with a computer-assisted image analysis system (Metamorph; Universal Imaging Co, Westchester, PA).^{17,24,38} Formalin-induced Fos staining was analyzed in the following 3 gray matter regions based on cytoarchitectonic criteria: (1) the superficial dorsal horn (SDH, laminae I and II); (2)

the nucleus proprius (NP, laminae III and IV); and (3) the neck region (NECK, laminae V and VI).

Evaluation of Side Effects

Systolic blood pressure (SBP) and heart rate (HR) were measured using a noninvasive computerized tail-cuff system (PowerLab system; ADI Instrument Pty Ltd, Chain Hills, NSW, Australia) as previously described.^{8,21} Briefly, animals were acclimated for 1 h in a quiet test room before obtaining cardiovascular measurements. At the end of the 1-hour period the SBP and HR were measured. This experiment was repeated 3 times and the mean value for each animal was recorded. Blood pressure and heart rates were recorded at 0, 10, and 30 minutes after treatment with clonidine. All cardiovascular measurements were obtained between 1 to 3 PM to avoid changes due to normal circadian rhythms.

We used a Rotorod test for mice and an open field test for rats, respectively¹⁸ to examine the potential sedative effects of clonidine, DBV or a combination of clonidine and DBV. The Rotorod apparatus (model# DJ-4009; Dae-Jong Engineering & Clean Technology, Seoul, Korea) consisted of a rotating horizontal bar (diameter = 6 cm), which was subdivided into 4 compartments by rotating plates. All mice were placed on the horizontal bar, which was set at a rotation speed of 4 revolutions per minutes. Twenty-four hours before the actual Rotorod test all mice were tested and those that were able to remain on the rod for at least 120 seconds were included in the study. Five minutes after clonidine injection, each animal was subsequently tested on the Rotorod over a 2-minute period and their performance time on the bar (in seconds) was measured. The test was repeated 3 times and the mean value for each animal was recorded.

In rats, the distance that a rat traveled during a 60-minute test period was measured using a spontaneous activity chamber (Model# SG-506; MED Associates, Georgia, VT). The activity test was initiated just after the administration of clonidine and was stopped 60 minutes later. Spontaneous ambulatory activity was determined in an open field (43 × 43 cm) Plexiglas box with height of 30 cm, equipped with infra-red photocells located in the walls 2 cm above a grid floor. Ambulatory activity was expressed as the distance traveled, calculated on the basis of the number of interruptions of the photobeams.

Statistical Analysis

Results are expressed as mean ± SE unless otherwise stated. Data analysis and statistical comparisons were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Comparison between 2 groups was made using the Student's *t*-test. For multiple comparisons, analysis of variance (ANOVA) followed by post hoc Newman-Keuls test was performed. For analysis of pain sensitivity in the CCI model at the different time points under different treatment conditions, the data were analyzed using a 2-way repeated-measures ANOVA followed by a post hoc Bonferroni analysis. Differences with *P* < .05 were considered significant.

Results

Effect of Apipuncture Pretreatment on Clonidine-Induced Antinociceptive Effects in the Mouse Formalin Test

Intrathecal clonidine injection produced a dose-dependent analgesic effect on formalin-induced nociceptive responses during both the early and late phases of the formalin test (Fig 1, A and B). By contrast injection of 0.01 mg/kg DBV into the Zusanli acupoint produced a slight decrease in formalin-induced nociceptive responses during the late but not the early phase of the formalin test (data not shown). Interestingly, injection of 0.01 mg/kg DBV into the Zusanli acupoint before i.t. clonidine injection caused a 3.5-fold leftward shift in the dose-response curve for clonidine during the early phase (ED 50: clonidine = 0.52 nmol vs clonidine plus DBV = 0.14 nmol) and a 5-fold shift in the dose-response curve for

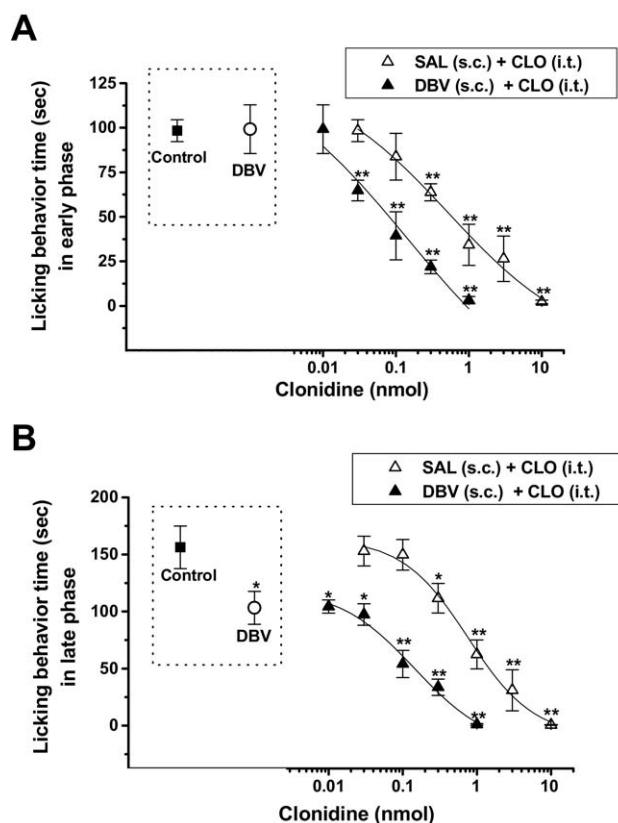


Figure 1. Graphs illustrating the antinociceptive effect of intrathecal (i.t.) clonidine alone or in combination with subcutaneous (s.c.) injection of diluted bee venom (DBV) into the Zusanli acupoint on formalin-induced nociceptive behavior during the early (A) and late (B) phases of the formalin test. Mice were intrathecally injected with saline (SAL) or clonidine (CLO) 5 minutes after s.c. injection of saline or DBV. DBV pretreatment caused a potent 3.5-fold (ED 50: clonidine vs clonidine plus DBV = 0.52 nmol vs 0.14 nmol) or a 5-fold (ED 50: 0.76 nmol vs 0.15 nmol) leftward shift of the clonidine dose response curve for antinociception during the early and late phases, respectively, of the formalin test. **P* < .05, ***P* < .01, significantly different from the control [SAL (s.c.) + SAL (i.t.)] group values. *n* = 7 mice per group.

clonidine during the late phase (ED 50: clonidine = 0.76 nmol vs clonidine plus DBV = 0.15 nmol). On the other hand, acupoint treatment with 0.001 mg/kg DBV in conjunction with 1 nmol clonidine did not potentiate the clonidine-induced antinociceptive effect (data not shown).

Effect of Combined i.t. Clonidine and Apipuncture Treatment on Formalin-Induced Spinal Cord Fos Expression

Formalin injection into the hind paw significantly increased Fos expression in spinal cord laminae I–VI (Fig 2A) as previously described. Clonidine administered at a dose of 0.1 nmol did not alter formalin-induced spinal Fos

expression. Subcutaneous injection of 0.01 mg/kg DBV into the Zusanli acupoint significantly decreased the formalin injection-induced expression of FLI (Fig 2, A and B) in the spinal cord dorsal horn. Moreover, when clonidine injection was combined with DBV apipuncture treatment, the suppression of formalin-induced FLI in the spinal cord dorsal horn was substantially greater than that produced by apipuncture alone (Fig 2A).

Effect of Combined Clonidine and Apipuncture Treatment on Neuropathic Pain-Induced Thermal Hyperalgesia and Mechanical Allodynia

Thermal Hyperalgesia

Before CCI surgery the mean value of the thermal stimulus-induced PWL was 11.06 ± 0.57 seconds. Animals who underwent CCI surgery showed significant thermal hyperalgesic responses at 14 days after surgery, at which point the mean PWL of the affected hind limb was significantly reduced to 7.01 ± 0.7 seconds. Fig 3A shows the antihyperalgesic effect of i.t. clonidine alone or in combination with DBV (0.25 mg/kg) apipuncture on PWL. Intrathecal treatment with clonidine dose-dependently suppressed thermal hyperalgesia. In particular i.t. injection of the highest dose (50 nmol) of clonidine produced a maximal antinociceptive effect on thermal hyperalgesia, whereas the lowest dose (5 nmol) tested in this study had no effect on CCI-induced thermal hyperalgesia. It is also important to point out that DBV injection into the Zusanli acupoint without clonidine treatment also produced a moderate, but significant, increase in PWL at the 15 and 30 minutes (after DBV injection) time points as compared to the control group (subcutaneous saline + i.t. saline). Although i.t. injection of 5 nmol clonidine had no effect on CCI-induced thermal hyperalgesia, when i.t. injection of 5 nmol clonidine was paired with DBV apipuncture pretreatment, it significantly increased PWL throughout the 60-minute post-DBV injection period. Moreover, apipuncture pretreatment combined with the 5 nmol dose of clonidine reversed the CCI-induced decrease in PWL back to its original pre-CCI baseline value at both the 30- and 60-minute time points after DBV injection. Additionally, whereas i.t. injection of 15 nmol clonidine alone significantly increased PWL only at the 15-minute time point compared to the control group, when injection of this same dose of clonidine was paired with DBV apipuncture treatment, it significantly increased PWL at the 15-, 30-, and 60-minute post-DBV time points. This paired effect was similar to that produced by i.t. injection of 50 nmol clonidine without DBV treatment. However, it is important to point out that when an i.t. injection of 15 nmol clonidine was paired with a subcutaneous injection of a 10-fold lower dose of DBV (0.025 mg/kg) into the Zusanli acupoint, there was a failure of the DBV to potentiate the clonidine-induced analgesic effect on CCI-induced thermal hyperalgesia (data not shown).

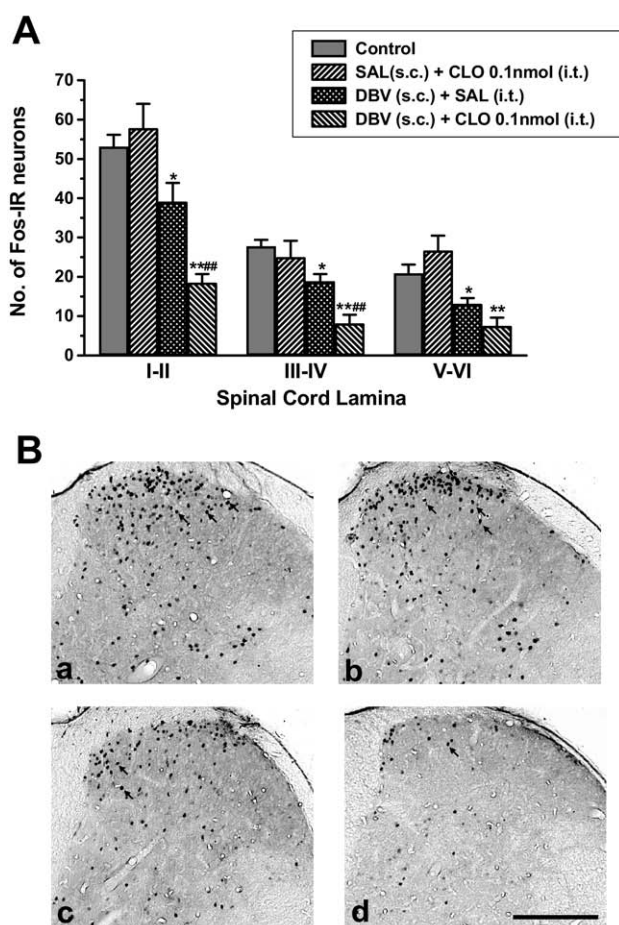


Figure 2. A, Graph illustrating the number of Fos-positive neurons in the ipsilateral spinal cord dorsal horn (L3–L5) induced by injection of formalin in the control and each of the 3 treatment groups ($n = 5$ mice per group). Mice were intrathecally (i.t.) injected with saline or clonidine (CLO, 0.1 nmol) 5 minutes after subcutaneous (s.c.) injection of saline (SAL) or diluted bee venom (DBV) into the Zusanli acupoint. SDH, superficial dorsal horn (laminae I–II); NP, nucleus proprius (laminae III–IV); NECK, neck of dorsal horn (laminae V–VI). $###P < .01$, significantly different from DBV (s.c.) + SAL (i.t.) group value. $**P < .01$: different from the number of Fos-labeled neurons in the Control (Saline (s.c.) + Saline (i.t.)) group. B, Representative photomicrographs of Fos protein immunostaining (black arrows) in spinal cord sections from each experimental and control group ($n = 5$ mice per group). a, control; b, SAL + CLO, 0.1 nmol; c, DBV + SAL; d, DBV + CLO, 0.1 nmol. Scale bar = 200 μ m.

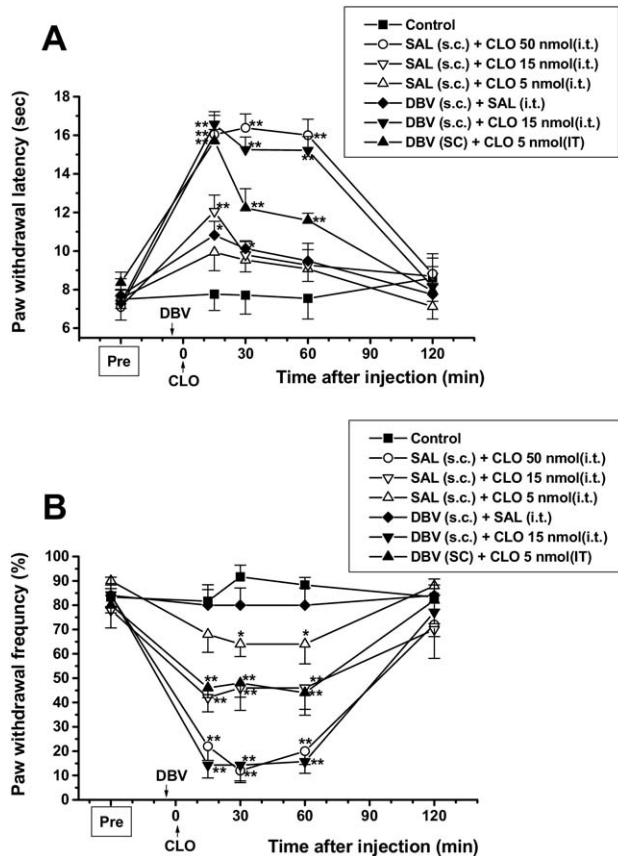


Figure 3. Graphs demonstrating the analgesic effects of intrathecal (i.t.) clonidine alone or in combination with an injection of subcutaneous (s.c.) diluted bee venom (DBV) on CCI-induced thermal hyperalgesia (A) or mechanical allodynia (B). Rats were injected i.t. with saline (SAL) or clonidine (CLO) 5 minutes after s.c. injection of saline or DBV. DBV pretreatment significantly potentiated clonidine-induced analgesia $*P < .05$, $**P < .01$, significantly different from the control [SAL (s.c.) + SAL (i.t.)] group values. $n = 7$ rats per group.

Mechanical Allodynia

Using an innocuous mechanical stimulus the mean basal paw withdrawal frequency (PWF) was $7.06\% \pm 1.24\%$ before CCI surgery. At 14 days after CCI, the mean PWF in the affected paw was significantly increased to $82.42\% \pm 2.34\%$, which is comparable to that previously reported. No statistically significant differences were detected between the values for PWF before and after apipuncture treatment with DBV (0.25 mg/kg) (Fig 3B). These results indicate that DBV stimulation of the Zusanli acupoint can reduce CCI-induced thermal hyperalgesia but not CCI-induced mechanical allodynia. As illustrated in Fig 3B, clonidine dose dependently decreased PWF. The highest dose (50 nmol) of clonidine produced the maximal antiallodynic effect on PWF. Pretreatment with DBV apipuncture stimulation potentiated the clonidine-induced decrease in PWF. In this regard, pretreatment with DBV caused the antiallodynic effect produced by i.t. injection of 15 nmol clonidine to mimic the effect produced by an i.t. injection of the 50-nmol dose of

clonidine alone. Similarly, pretreatment with DBV apipuncture caused the 5-nmol dose of clonidine to produce an effect that was similar to that produced by 15 nmol clonidine alone. Administration of a 10-fold lower dose (0.025 mg/kg) of DBV combined with 15 nmol clonidine was ineffective in modifying the analgesic effect produced by 15 nmol clonidine alone on mechanical allodynia (data not shown).

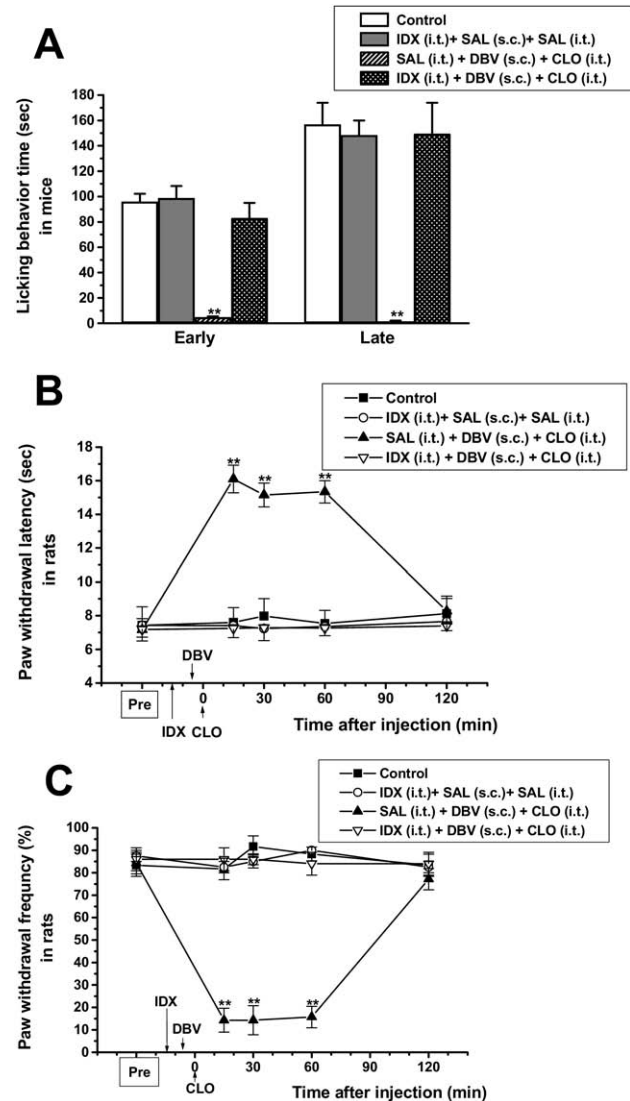


Figure 4. Graphs illustrating the effects of the α_2 -adrenoceptor antagonist, idazoxan on the enhanced analgesic effect produced by i.t. clonidine (CLO) in combination with an injection of diluted bee venom (DBV) into the Zusanli acupoint. Animals were pretreated with intrathecal (i.t.) idazoxan (IDX, 10 μ g per mouse, 50 μ g per rat) 10 minutes before subcutaneous (s.c.) DBV injection, which was followed by i.t. clonidine (1 nmol per mouse, 15 nmol per rat). Pretreatment with IDX potently reversed the antinociceptive effect of clonidine combined with DBV treatment on formalin-induced nociceptive behavior ($n = 7$ mice per group) (A) and on CCI-induced thermal hyperalgesia (B) and mechanical allodynia (C) ($n = 7$ rats per group). $**P < .01$, significantly different from control [SAL (i.t.) + SAL (s.c.) + SAL (i.t.)] group value.

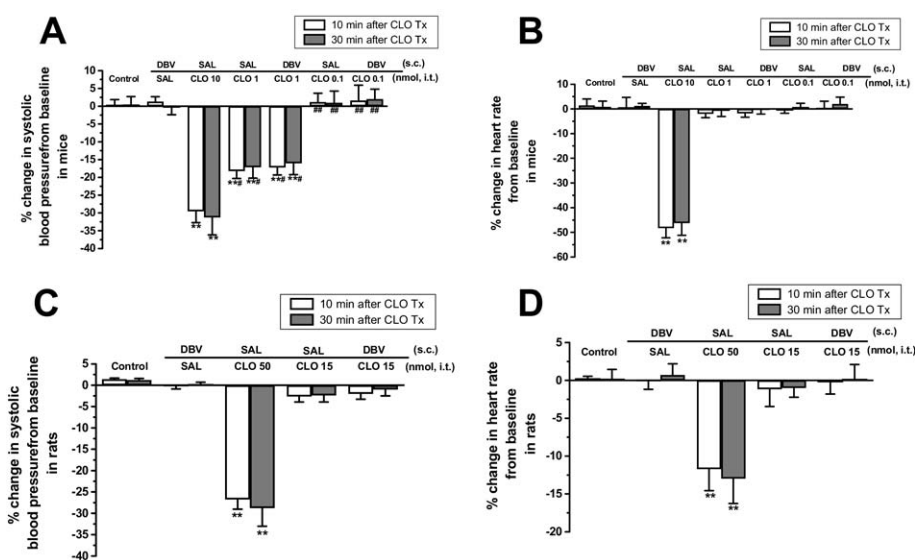


Figure 5. Graphs demonstrating the effect of intrathecal (i.t.) injection of clonidine (CLO) together with subcutaneous (s.c.) injection of diluted bee venom (DBV) into the Zusanli point on blood pressure (**A and C**) and heart rate (**B and D**) in mice (**A and B**) and in rats (**C and D**). Intrathecal injection of a high dose of clonidine (10 nmol for mice, 50 nmol for rats) induced significant hypotension and bradycardia. SAL, saline. ** $P < .01$ significantly different from the Control [SAL (s.c.) + SAL (i.t.)] group values. ## $P < .01$ significantly different from SAL (s.c.) + CLO 10 nmol (i.t.) group values. $n = 7$ per group.

The Role of Spinal α_2 -Adrenoceptors in the DBV Apipuncture-Induced Potentiation of Clonidine's Analgesic Effect

With respect to the neuropathic pain model used here, we have previously reported that administration of the α_2 -adrenoceptor antagonist, idazoxan (50 μ g) to CCI rats is sufficient to inhibit the analgesic effects produced by either DBV apipuncture or by administration of a 40 μ g dose of clonidine.^{37,39} Moreover, in the formalin test, administration of a 10- μ g dose of idazoxan is sufficient to totally block the DBV-induced or high dose clonidine-induced antinociceptive effect as shown in a previous report³⁸ and as verified in the current study (data not shown). Therefore, these specific doses of idazoxan were selected for mice and rats in the present study to investigate the role of spinal α_2 -adrenoceptors in the DBV apipuncture-induced potentiation of clonidine's analgesic effect. In the mouse formalin test, the enhanced antinociceptive effect produced by the coadministration of 1 nmol clonidine with DBV apipuncture was completely blocked by preadministration of idazoxan (Fig 4A). Similarly, in the rat neuropathic pain model, i.t. idazoxan reversed the analgesic effect produced by 15 nmol clonidine in conjunction with DBV apipuncture on both thermal hyperalgesia (Fig 4B) and mechanical allodynia (Fig 4C).

Treatment Side Effects on Blood Pressure, Heart Rate, Motor Performance, and Locomotor Activity

The baseline systolic blood pressure and heart rate were 120.8 ± 4.7 mm Hg and 612.6 ± 16.6 BPM for mice and 111.0 ± 3.6 mm Hg and 323.0 ± 5.1 BPM for rat,

respectively. As shown in Fig 5, DBV alone did not affect systolic blood pressure (Fig 5, A and C) or heart rate (Fig 5, B and D) in either mice or rats. In mice, i.t. clonidine (10 nmol) significantly decreased systolic blood pressure (Fig 5A) and heart rate (Fig 5B) measured 10 and 30 minutes after clonidine treatment. Clonidine given at a dose of 1 nmol also decreased systolic blood pressure, but this effect was smaller than that produced by the 10-nmol dose of clonidine. Moreover, the 1 nmol-dose of clonidine did not affect heart rate. Additionally, the lowest dose of clonidine (0.1 nmol) had no effect on either systolic blood pressure or heart rate. In CCI rats, clonidine (50 nmol) significantly decreased systolic blood pressure and heart rate. However, there were no changes in heart rate or blood pressure after treatment with 15 nmol clonidine alone or when 15 nmol clonidine was coadministered with DBV apipuncture. On the other hand, in the mouse Rotorod test (Fig 6A), DBV or saline injection into the Zusanli acupoint did not affect motor coordination. Intrathecal injection of 10 nmol clonidine significantly decreased motor function compared with that of the i.t. saline treatment group, whereas neither 1 nmol clonidine nor its combination with DBV apipuncture affected the motor function score. In rat open field test (Fig 6B), clonidine given at a dose of 50 nmol greatly decreased locomotor activity indicating a significant sedative effect of this high dose of clonidine. However, neither 15 nmol clonidine alone nor 15 nmol clonidine combined with DBV apipuncture had any significant effect on locomotor activity.

Discussion

In this study, pretreatment with apipuncture, a chemical form of acupuncture in which DBV is subcutaneously

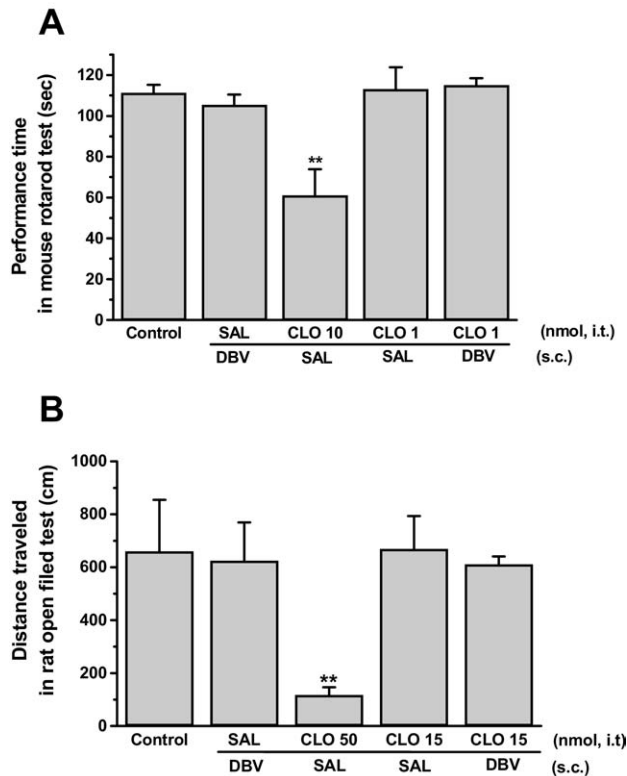


Figure 6. Graphs demonstrating the combined effect of intrathecal (i.t.) clonidine (CLO) treatment and subcutaneous (s.c.) injection of diluted bee venom (DBV) into the Zusanli acupoint on motor performance in mice (A) or locomotor activity in rats (B). The high dose of clonidine (10 nmol for mice, 50 nmol for rats) significantly decreased motor performance or locomotor activity, respectively, whereas DBV alone or in combination with low dose of clonidine had no effect. SAL, saline, ** $P < .01$ significantly different from the control [SAL (s.c.) + SAL (i.t.)] group values. $n = 7$ per group.

injected into an acupoint, was found to potentiate clonidine-induced analgesia in both the mouse formalin test and the rat CCI neuropathic pain model. Previous studies from our laboratories have also demonstrated that DBV injection into the Zusanli acupoint significantly reduces hyperalgesia in the formalin test, in a rat model of arthritis, and in a rat model of neuropathic pain, compared to non-acupoint (arbitrary site of back) stimulation, which is ineffective.^{17,23,37} Based on these data the Zusanli-acupoint was selected in the present study to evaluate the potential enhancing effect of DBV apupuncture on clonidine-induced analgesia.

In the mouse formalin test, DBV (0.01mg/kg) injected into the Zusanli acupoint was found to suppress formalin-induced licking behavior during the late phase, but not the early phase, confirming a recent report from our laboratories.³⁸ Although we have previously demonstrated that a high dose of DBV (10 mg/kg) produced a significant antinociceptive effect on both the early and late phases of the formalin test, this high dose of DBV also induces activation of nociceptive neurons and thus induces some degree of nociception at the site of injection.³⁸ Therefore a high dose of DBV was not used in the

present studies. Additionally, i.t. clonidine was shown in the present study to dose-dependently suppress formalin-induced nociceptive behavior in both the early and late phases, which is in agreement with previous studies.^{15,34,50} However, the maximal effective dose of clonidine is typically accompanied by significant side effects that include hypotension, bradycardia, lethargy and weakness, which serve to limit the use of clonidine particularly at higher, clinically effective doses.²⁰ An important finding in the present study was that DBV apupuncture treatment produced a leftward shift in the clonidine dose-response curve during both the early (0 to 10 minutes) and late (10 to 30 minutes) phases of the formalin test. Importantly, pairing low-dose clonidine treatment with DBV apupuncture did not induce any changes in blood pressure, heart rate or motor activity, which is consistent with the use of this combination to treat pain without the unwanted side effects of typical higher doses of clonidine.

It is important that combined DBV apupuncture and low dose clonidine were able to block both the early phase and late phase responses in the formalin test. As mentioned above, the early phase response of the formalin test is caused by the direct stimulation of nociceptors by formalin or tissue damage and is thought to be an acute pain reaction.⁴⁸ The late phase response is caused by subsequent inflammation after formalin injection and central sensitization related to C-fiber activity.^{30,48} Therefore, our results indicate that DBV apupuncture is able to potentiate the clonidine-induced suppressive effect on not only acute nociceptive activity, but also on inflammation-induced central sensitization. These findings are reinforced by the spinal Fos expression data. It is generally well accepted that increases in nociceptive stimulus intensity (eg, formalin in this study) produce corresponding increases in dorsal horn Fos expression.^{4,12,17,38} The dramatic reduction in formalin-induced Fos staining in all laminae of the dorsal horn in the mice receiving low dose clonidine paired with apupuncture are consistent with the behavioral data. Systemic clonidine, particularly higher doses of clonidine, have been shown to activate Fos in dorsal horn neurons.¹⁰ Our results are consistent with this, since there were slightly more Fos positive neurons in laminae I, II, V, and VI in formalin-injected mice treated with 0.1 nmol clonidine than controls in the present study. However the numbers of Fos neurons in the dorsal horn dropped dramatically in formalin injected mice receiving paired DBV/clonidine treatment, which is supportive of this combined therapeutic approach to treat acute pain conditions.

However, transient pain is distinctly different from persistent pain, which is associated with long-lasting alterations of the nervous system,³⁶ and thus findings from acute animal nociceptive models including the formalin test may not apply to more persistent pain states. Therefore, we also tested the effect of combined apupuncture and intrathecal clonidine injection on CCI-induced neuropathic pain. In this model, DBV (0.25 mg/kg) apupuncture alone moderately reduced CCI-induced thermal hyperalgesia, but had no effect on mechanical allodynia

similar to that of a previous report.³⁷ However, when combined with DBV apipuncture, i.t. clonidine significantly potentiated the analgesic effect on both thermal hyperalgesia and mechanical allodynia without any side effects.

We have previously reported that the DBV apipuncture-induced antinociceptive effect is blocked by i.t. pretreatment with α_2 -adrenoceptor antagonists in several different rodent pain models, indicating that this DBV-induced antinociceptive effect is mediated by spinal α_2 -adrenoceptors.^{19,22,25,38} Moreover, it is well established that i.t. clonidine also produces its analgesic effect via direct activation of spinal α_2 -adrenoceptors.^{2,6,7,45} Therefore, we hypothesized that DBV apipuncture enhances the antinociceptive effect of clonidine via a functional potentiation of spinal α_2 -adrenoceptors. In support of this assumption, the enhanced analgesic effect produced by the combination of clonidine and DBV apipuncture treatment was totally blocked by pretreatment with the α_2 -adrenoceptor antagonist, idazoxan, in both the formalin test and the CCI neuropathic pain model. Interestingly, this DBV apipuncture did not significant side effects like that of i.t. clonidine, although the antinociceptive effect of DBV is also mediated by spinal α_2 -adrenoceptors. Because of its high lipophilicity,⁴⁷ i.t. clonidine is well absorbed systemically and thus induces nonspecific side effects such as hypotension, bradycardia, sensorimotor deficits and sedation.^{5,16,33,43} However, this DBV-induced analgesic effect appears to be mediated by activation of specific neuronal pathways and not by systemic absorption of DBV.^{3,26} In this regard, several lines of evidence support the concept that DBV-induced apipuncture leads to activation of brainstem noradrenergic projection neurons, which in turn activate spinal α_2 -adrenoceptors via descending bulbospinal pathways.^{24,38} Moreover, lidocaine-induced blockage of sciatic nerve conduction causes a complete suppression of DBV-induced neuronal firing in the spinal cord dorsal

horn suggesting DBV-induced specific neuronal conduction.³ Collectively, the results of the present study suggest that the enhanced analgesic effect produced by clonidine in conjunction with DBV apipuncture treatment is due to the selective activation of α_2 -adrenoceptors in the spinal cord dorsal horn and not to activation of these receptors either peripherally or at supraspinal sites. Recent work has shown that nerve injury causes sprouting of spinal noradrenergic fibers and that this sprouting paradoxically increases the capacity of adrenergic drugs to produce analgesia in the setting of neuropathic pain.¹³ If this occurs in the CCI model used here, then the enhanced affect of low dose clonidine and DBV injection could be attributed to their effects on increased α_2 -adrenoceptor location or function resulting from injury induced noradrenergic sprouting observed under conditions of neuropathic pain.

In conclusion, the current study demonstrated that pretreatment with DBV apipuncture consistently potentiated clonidine-induced analgesia in an acute formalin-induced pain model and in a more chronic neuropathic pain model. The highest dose of clonidine (maximal antinociceptive dose) was associated with the development of severe side effects. However, when lower doses of clonidine were combined with apipuncture the antinociceptive effect obtained was equivalent to that of the high dose of clonidine but without significant side effects. Moreover, this enhanced analgesic effect was completely blocked by pretreatment of an α_2 -adrenoceptor antagonist. Our findings indicate that pairing a lower dose of an α_2 -adrenoceptor agonist, like clonidine, with acupuncture stimulation may provide an improved strategy for pain management, by enhancing analgesia while decreasing α_2 -adrenoceptor agonist-induced side effects. Thus this combined therapeutic approach decreases the side effects of drug therapy and suggests a possibility of novel strategy for pain management.

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