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COMPARISON OF ANALGESIC EFFECTS BETWEEN SEROTONIN AND EPIBATIDINE WHEN COMBINED WITH CLONIDINE BY INTRATHECAL ADMINISTRATION IN RAT MODELS

***Tomoki Nishiyama MD, PhD**

Department of Emergency Medicine (Anesthesiology), Maruyama Memorial General Hospital, 2-10-5, Hon-cho, Iwatsuki-ku, Saitama-shi, Saitama, 339-8521, Japan.

***Corresponding Author: Dr. Tomoki Nishiyama MD, PhD**
Department of Emergency Medicine (Anesthesiology), Maruyama Memorial General Hospital, 2-10-5, Hon-cho, Iwatsuki-ku, Saitama-shi, Saitama, 339-8521, Japan.

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ABSTRACT

Background: The present study was performed to compare analgesic effects of the combination of intrathecal clonidine and serotonin with that of clonidine and epibatidine to know whether serotonin or epibatidine is better to combine with clonidine in intrathecal analgesia. **Methods:** Male Sprague-Dawley rats implanted with lumbar intrathecal catheters were given intrathecal combination of clonidine with serotonin or epibatidine, then tail flick test or formalin test was performed. Isobolographic analysis was done using 50 % effective doses, and total fractional dose values were calculated. **Results:** Tail flick latency increased with clonidine + serotonin compared to the control. Higher doses of clonidine + epibatidine increased tail flick latency, while lower doses decreased it. Clonidine + serotonin showed synergistic effect in the tail flick test. Clonidine + epibatidine showed additive effect in the tail flick test. The number of flinches in the formalin test decreased with clonidine + serotonin, and clonidine + epibatidine, but not dose dependent. Synergistic effects were observed in both phases of the formalin test with clonidine + serotonin. Additive effect in the phase 1 and antagonistic effect in the phase 2 were observed in the formalin test with clonidine + epibatidine. **Conclusions:** For acute thermal and acute and chronic inflammatory pain, clonidine + serotonin might be better than clonidine + epibatidine when intrathecally administered.

KEYWORDS: nicotinic acetylcholine receptor, epibatidine, serotonin, α2-adrenoceptor, clonidine, intrathecal, analgesia.

INTRODUCTION

There are many mechanisms in the spinal cord regulating pain transmission. We have already shown that epibatidine, a nicotinic acetylcholine (nACh) receptor agonist[1,2], serotonin, a 5-hydroxytriptamine (HT) receptor agonist[3], and clonidine, a α_2 receptor agonist[4], had analgesic effects when administered intrathecally in rat. However, these agents induce some side effects and toxic effects. To decrease adverse effects, synergistic combination is preferable. The present study was performed to compare analgesic effects of the combination of intrathecal clonidine and serotonin with that of clonidine and epibatidine to know whether serotonin or epibatidine is better to combine with clonidine in intrathecal analgesia.

MATERIALS AND METHODS

After obtaining the approval of the Research Committee of the University of Tokyo, male Sprague-Dawley rats (280-300 g; Nippon Bio-Supply, Tokyo, Japan) were implanted with lumbar intrathecal catheters under halothane (2 %) anesthesia. The experiment procedures are the same as our previous study.[4] Briefly, an 8.5 cm polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ) was inserted caudally to the thoracolumbar level in the intrathecal space through atlanto-occipital membrane. The rostral part of the catheter was plugged with a 28-gauge steel wire and put through to the top of the skull. Only rats with normal motor function and behavior and increase in body weight seven days later were used for experiments. After the study, rats were euthanized under halothane 5% and the location of the catheter was confirmed anatomically and the data of the rats with mal location of the catheter was excluded, and another rat was added to fill the number of each group.

Drug preparation

Combination of each 1/2, 1/4, 1/8, or 1/16 50% effective doses (ED50s) of serotonin[3] (serotonin receptor agonist, Sigma, St. Louis, MO) + clonidine (α_2 receptor agonist, Sigma)[4], or epibatidine (nACh receptor agonist,

Sigma)[2] + clonidine were dissolved in 10 μL using saline.

Nociceptive test

According to our previous study[4], we used the same two classic methods as nociceptive tests.

Tail-flick test

The tail-flick test was performed with the Tail-Flick Analgesia Meter (MK-330A; Muromachi Kikai Co. Ltd., Tokyo, Japan). Rats were placed in a clear plastic cage with their tails extending through a slot located of the rear of the cage. Thermal stimulation was given by a beam of high intensity light focused on the tail 2 to 3 cm proximal to the end. The time between the start of the stimulation and tail withdrawal response was measured as a tail-flick latency. The cut-off time in the absence of a response was set to 14 seconds to prevent tissue injury of the tail. The test was done at 5, 10, 15, 30, 60, 90, 120, 180, and 240 minutes after drug injection. The data were shown as the % of maximum possible effect (% MPE): % MPE = (post-drug latency – pre-drug latency at time 0) X 100 / (cut-off time (14 sec) – pre-drug latency at time 0.

Formalin test

The formalin test was performed 10 minutes after intrathecal drug injection. Fifty μL of 5 % formalin was injected subcutaneously into the dorsal surface of the right hind paw with a 30 G needle. Immediately after injection, the rat was placed in an open clear plastic chamber and their flinching or shaking paw response was observed for 60 minutes. The number of flinches was counted for 1 minute. Usually two phases were observed: phase 1, during 0 to 6 minutes after formalin injection; and phase 2, beginning about 10 minutes after injection with the interval of no flinches between both phases.

Side effects

Side effects were examined and judged as present or absent. Agitation was judged as spontaneous irritable movement, vocalization, or both. Allodynia-like behavior was judged as escape, vocalization, or both induced by lightly stroking the flank of the rat with a small probe. The placing or stepping reflex was evoked by drawing the dorsum of either hind paw across the edge of the table. Normal rats try to put the paw ahead into a position to walk. The righting reflex was assessed by placing the rat horizontally with its back on the table. Normally rats twist the body to an upright position immediately. Flaccidity was judged as muscle weakness by putting the forepaw 3 to 5 cm higher than the hind paw. Normal rats will walk up. Pinna or corneal reflex was examined with a paper string. When a string is put into the ear canal or touches the cornea, rats normally shake their heads. Behavioral side effects were checked simultaneously with the tail flick test.

Protocol

After injection of each combination, the catheter was flushed with saline 10 μL to clear the dead space of the catheter. In each test, 8 rats were used.

The ED50s of the combination were obtained using the maximum effects in the tail flick test and the area under the curve of the number of flinches in the formalin test. To compare the theoretical additive point with experimentally derived ED50, isobolographic analysis was used. Total fractional dose values were calculated to describe the magnitude of the interaction as follows: (ED50 dose of drug 1 in combination) / (ED50 dose of drug 1 alone) + (ED50 dose of drug 2 in combination) / (ED50 dose of drug 2 alone). The value was normalized by assigning the ED50 value of each drug given alone as 1. Values near 1 suggest an additive interaction, values > 1 implies an antagonistic interaction, and values < 1 indicate a synergistic interaction.

Data analysis

The data are shown as mean ± standard deviation or 95% confidential interval (CI). Statistical analysis was performed with the analysis of variance (ANOVA) followed by Neuman-Keuls test to compare the calculated ED50 values with the theoretical additive values. A p value less than 0.05 was considered to be statistically significant.

RESULTS

Tail flick latency increased with clonidine + serotonin compared to the control (Fig.1).

Higher doses of clonidine + epibatidine increased tail flick latency, while lower doses decreased it (Fig.2). Clonidine + serotonin showed synergistic effect in the tail flick test (Fig.3, Table 1, 2). Clonidine + epibatidine showed additive effect in the tail flick test (Fig.4, Table 1, 2). The number of flinches in the formalin test decreased with clonidine + serotonin (Fig. 5), and clonidine + epibatidine (Fig. 6), but not dose dependent.

Synergistic effects were observed in both phases of the formalin test with clonidine + serotonin (Fig. 7, 8, Table 1, 2). Additive effect in the phase 1 (Fig. 9, table 1, 2) and antagonistic effect in the phase 2 (Fig. 10, Table 1, 2) were observed in the formalin test with clonidine + epibatidine.

In the combination of 1/2ED50s of clonidine + serotonin, one rat showed agitation and allodynia. No other behavioral side effects were observed.

Table 1. ED50

	Tail flick	Formalin phase 1	Formalin phase 2
Clonidine (μg)[1]	0.26 (0.16-0.42)	0.12 (0.07-0.20)	0.13 (0.07-0.25)
Serotonin (μg)[6]	34.4 (21.3-55.6)	12.6 (5.6-31.0)	1.3 (0.04-8.2)
Epibatidine (ng)[5]	32 (22.0-46.5)	38.0 (21.5-65.1)	27.1 (10.4-43.5)
Clonidine + Serotonin			
Clonidine (μg)	0.035 (0.028-0.042)	0.024 (0.013-0.045)	0.026 (0.012-0.038)
Serotonin (μg)	4.6 (3.7-5.6)	0.24 (0.13-0.45)	0.26 (0.12-0.38)
Clonidine + Epibatidine			
Clonidine (μg)	0.1 (0.03-0.37)	0.07 (0.01-0.28)	0.12 (0.03-0.77)
Epibatidine (ng)	12.4 (3.4-35.9)	13.9 (2.0-39.0)	24.6 (9.5-46.0)

Mean (95% confidence interval)

Table 2. Total dose fractional values.

	Tail flick	Formalin phase 1	Formalin phase 2
Clonidine + Serotonin	0.27 (0.20-0.35)	0.22 (0.21-0.24)	0.41 (0.22-3.15)
Clonidine + Epibatidine	0.77 (0.22-1.67)	0.95 (0.24-2.0)	1.83 (1.34-4.06)

Mean (95% confidence interval)

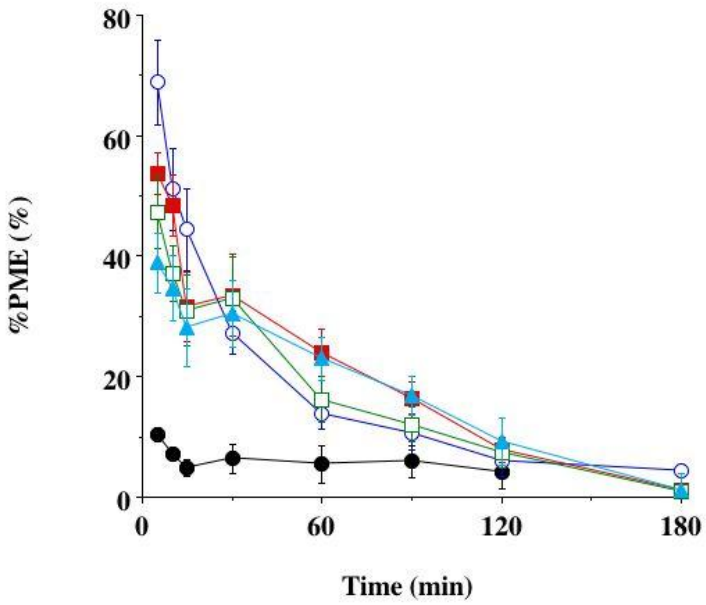


Fig.1 Tail flick latency of clonidine + serotonin.

%MPE, % maximum possible effect; Bars show standard deviation.

closed circle, saline; open circle, 1/2 ED50s; closed square, 1/4 ED50s; open square, 1/8 ED50s; closed triangle, 1/16 ED50s

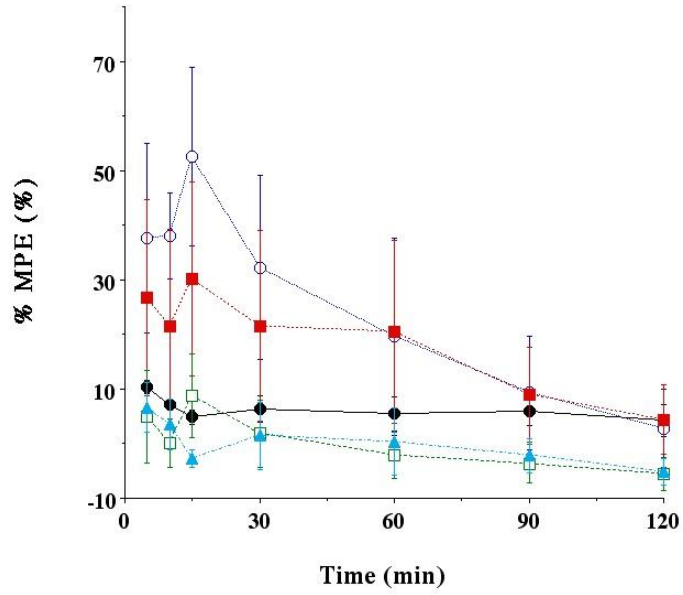
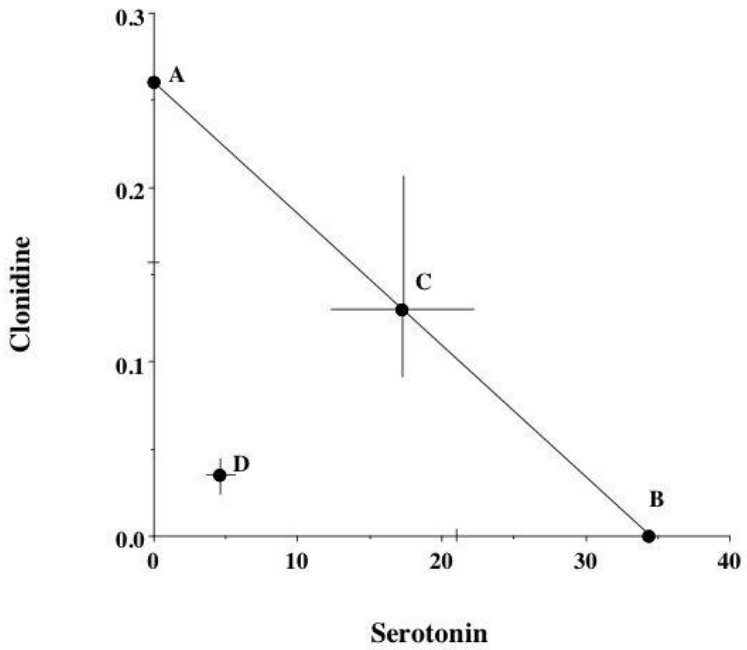


Fig.2 Tail flick latency of clonidine + epibatidine.

%MPE, % maximum possible effect; Bars show standard deviation.
closed circle, saline; open circle, 1/2 ED50s; closed square, 1/4 ED50s; open square, 1/8 ED50s; closed triangle, 1/16 ED50s.



Fig,3 Isobolograph of the tail flick test with clonidine and serotonin.

Y axis, µg; X axis, µg; A, ED50 of clonidine; B, ED50 of serotonin; C, theoretical additive point; D, ED50 of the combination Synergistic effect was observed.

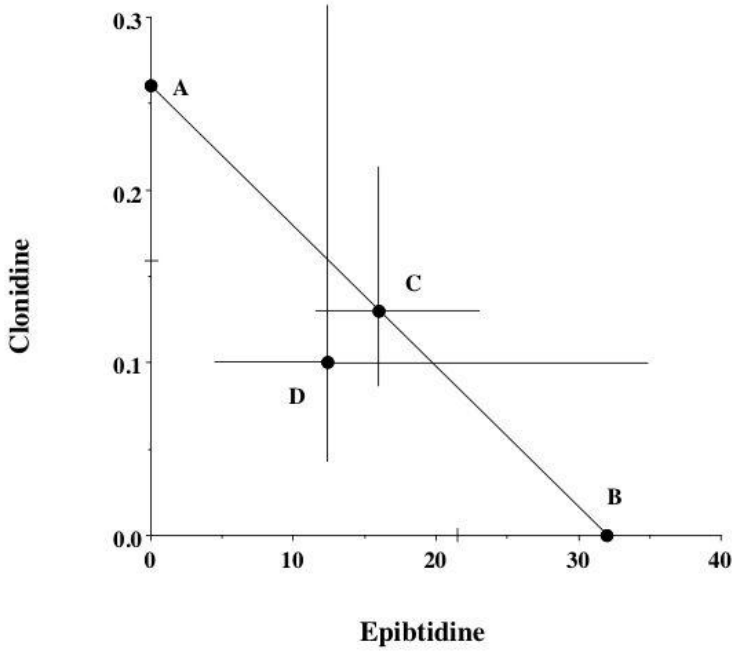


Fig.4 Isobolograph of the tail flick test with clonidine and epibatidine.

Y axis, μg; X axis, ng; A, ED50 of clonidine; B, ED50 of epibatidine; C, theoretical additive point; D, ED50 of the combination Additive effect was observed.

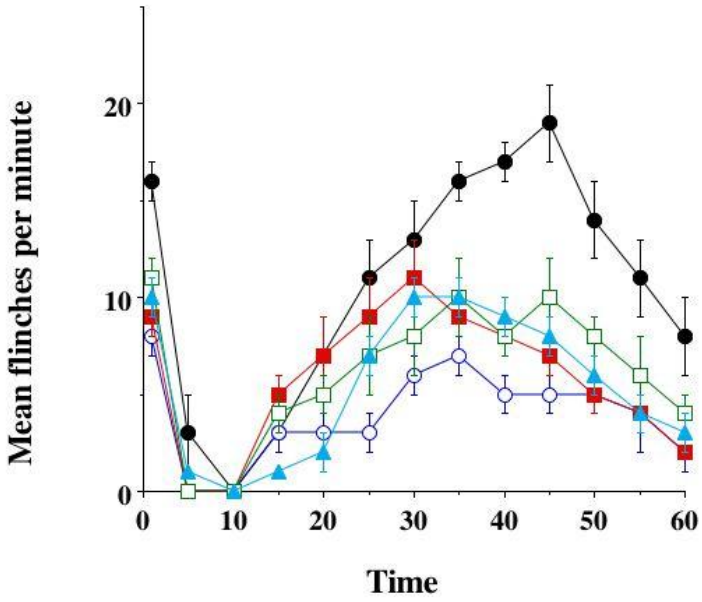


Fig.5 Flinch responses of the formalin test with clonidine and serotonin.

closed circle, saline; open circle, 1/2 ED50s; closed square, 1/4 ED50s; open square, 1/8 ED50s; closed triangle, 1/16 ED50s.

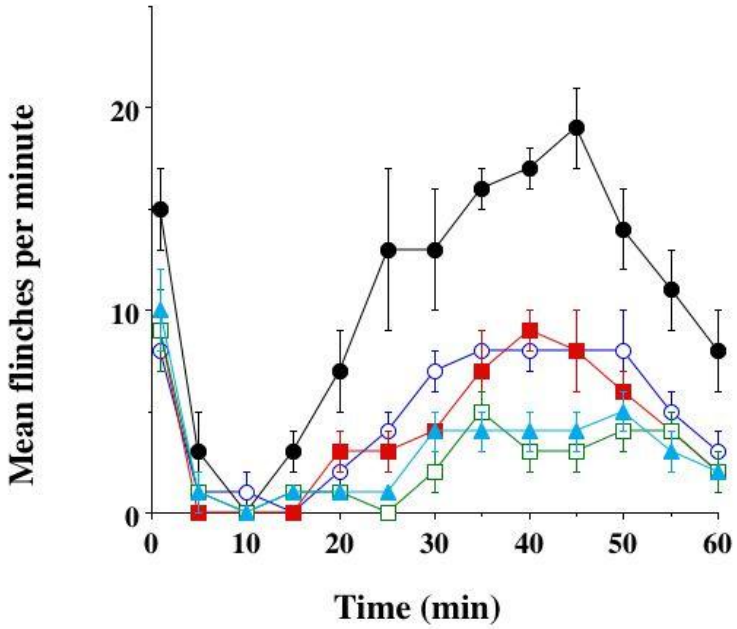


Fig. 6 Flinch responses of the formalin test with clonidine and epibatidine.

closed circle, saline; open circle, 1/2 ED50s; closed square, 1/4 ED50s; open square, 1/8 ED50s; closed triangle, 1/16 ED50s.

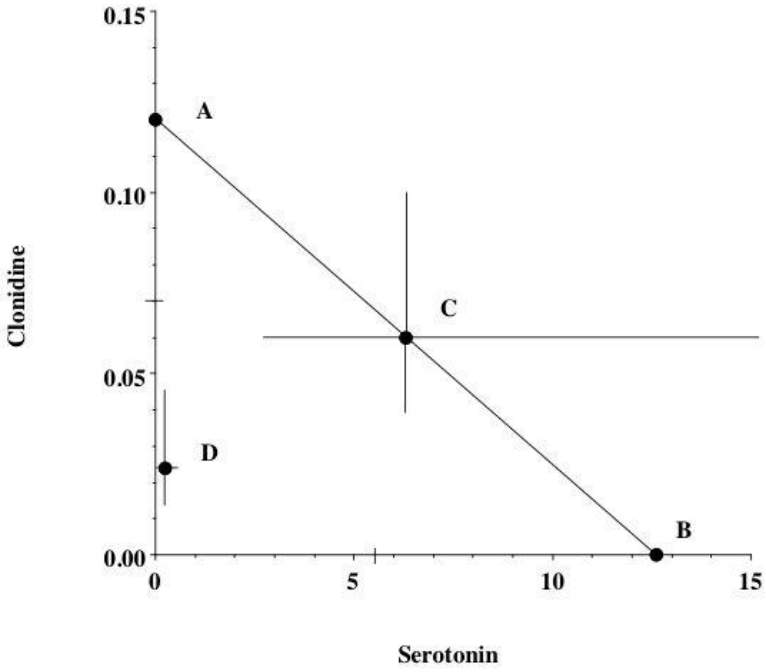


Fig. 7 Isobolograph of the formalin test phase 1 with clonidine and serotonin.

Y axis, μg; X axis, μg; A, ED50 of clonidine; B, ED50 of serotonin; C, theoretical additive point; D, ED50 of the combination Synergistic effect was observed.

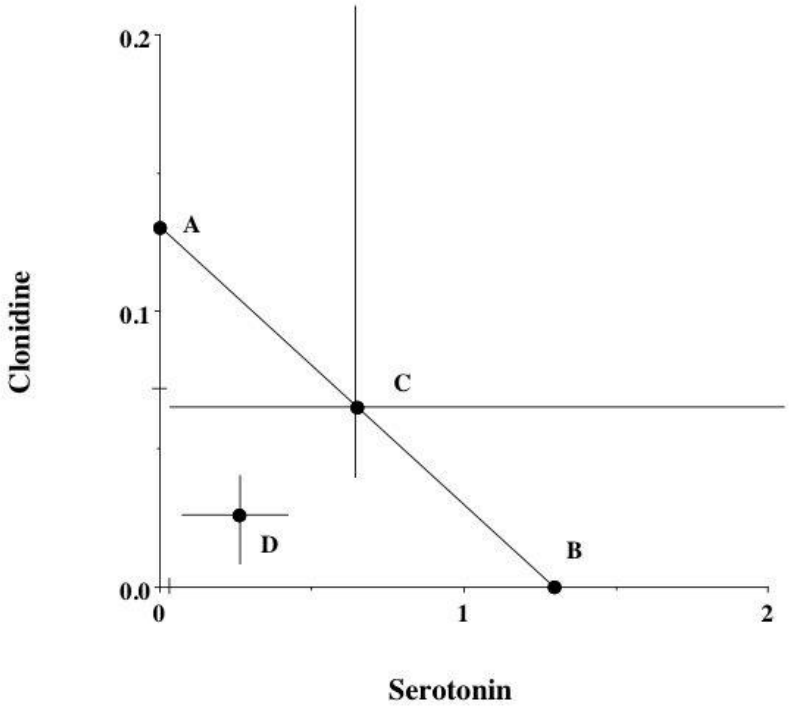


Fig. 8 Isobolograph of the formalin test phase 2 with clonidine and serotonin.

Y axis, µg; X axis, µg; A, ED50 of clonidine; B, ED50 of serotonin; C, theoretical additive point; D, ED50 of the combination Synergistic effect was observed.

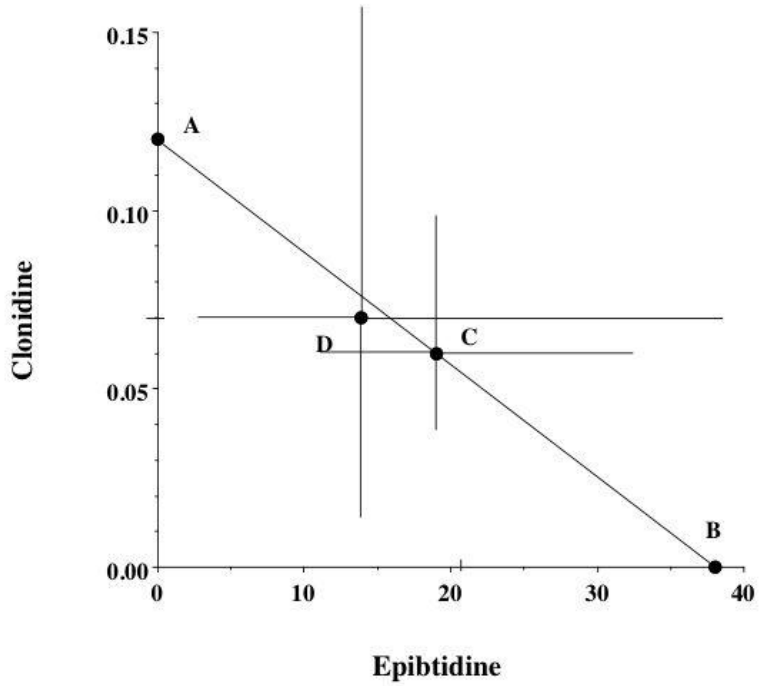


Fig. 9 Isobolograph of the formalin test phase 1 with clonidine and epibatidine.

Y axis, µg; X axis, ng; A, ED50 of clonidine; B, ED50 of epibatidine; C, theoretical additive point; D, ED50 of the combination Additive effect was observed.

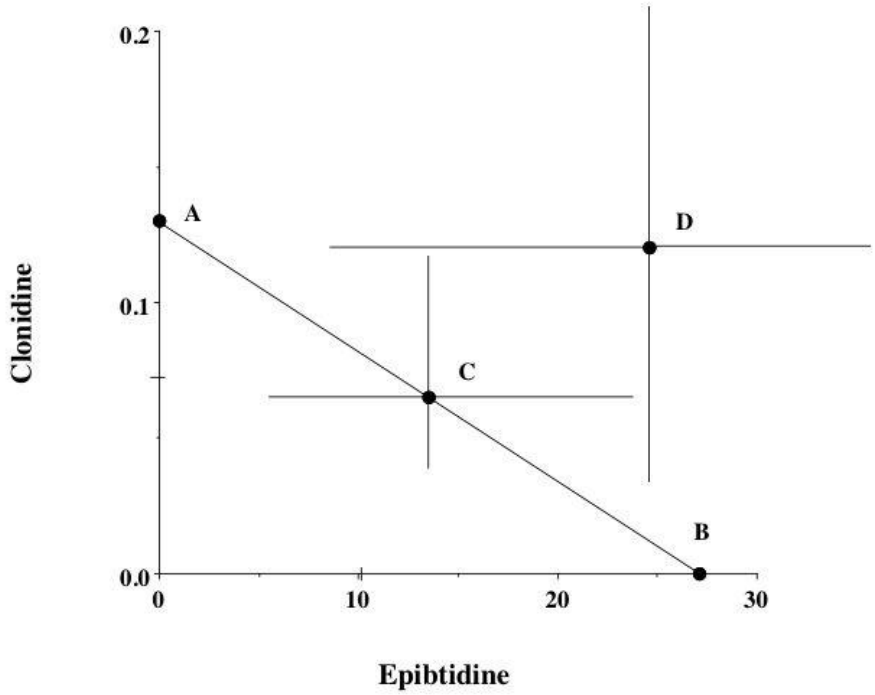


Fig. 10 Isobolograph of the formalin test phase 2 with clonidine and epibatidine.

Y axis, μg; X axis, ng; A, ED50 of clonidine; B, ED50 of epibatidine; C, theoretical additive point; D, ED50 of the combination Antagonistic effect was observed.

DISCUSSION

The present results showed that intrathecal clonidine + serotonin had synergistic analgesia for both acute thermal and inflammatory induced acute and chronic pain. However, intrathecal clonidine + epibatidine had only additive analgesia for acute thermal and inflammatory induced acute pain, but enhanced inflammatory induced chronic pain.

We used tail flick test and formalin test in this study because we have used these two methods in the previous studies, therefore, we could use the data of each agent from these previous studies[2,3,4] to save animals. The tail flick test is a model of acute thermal pain, and the formalin test is a model of inflammatory pain with the phase 1 as acute pain and phase 2 associated with central hypersensitivity, chronic pain.

Epibatidine stimulates nACh receptors.[5] Intrathecal nACh receptor agonists produce both analgesic and algesic behaviors via stimulation of separable populations of nACh receptors.[6] The algesic effects of intrathecal epibatidine are mediated by α_7 nACh receptors, but analgesic effects are mediated by different subtypes of nACh receptors.[6] However, Rowley et al.[7] reported that α_7 nACh receptor activation might provide analgesia after injury. Therefore, different kinds of nACh receptors showed different effects according to the kinds of pain stimuli. Intrathecal epibatidine inhibited development of hyperalgesia and inflammation[8] and showed analgesic effect on thermal induced acute pain.[1]

Nicotinic agonists were shown to potentiate inhibitory synaptic transmission in many different layers of the spinal cord.[9] NAchR agonists activate cholinergic, noradrenergic, and serotonergic neurons and enhances the release of Ach, noradrenaline, and serotonin.[10] Spinal nACh receptors mediate nitric oxide release caused by activation of α_2 receptors in normal and neuropathic rats.[11] Clonidine increases acetylcholine (ACh) in the dorsal horn in the spinal cord.[12] Both α_2 and ACh receptors co-localize in the dorsal horn of the spinal cord.[13] Analgesic effects of clonidine are associated with release of ACh in the dorsal horn of the spinal cord.[14] Clonidine also increased GABA in vivo in the spinal cord of spinal nerve ligation rats, which was blocked by α_2 adrenergic and nicotinic cholinergic antagonists.[15] Therefore, epibatidine might increase the effects of clonidine.

The combination of intrathecal clonidine and epibatidine dose -dependently reduced tonic pain behaviors.[16] Clonidine decreases release of glutamate and substance P from primary afferent nerve terminals[17], hyperpolarizes dorsal horn wide dynamic range neurons.[18] However, intrathecal nACh receptor agonists induced excitatory amino acid release and produced pain behaviors blocked by NMDA receptor antagonists.[19] Therefore, combination of clonidine and epibatidine might show

either algesic or analgesic effects. Our results showed additive effects of intrathecal clonidine + epibatidine in acute thermal and acute inflammatory pain, but antagonistic effects on chronic inflammatory pain.

Serotonin acts on different subtypes of 5-HT receptors in the spinal cord. Intrathecal 5-HT_{1A} receptor agonist increased hyperalgesia.[20] Other 5-HT_1 receptors are involved in analgesic mechanisms in the spinal cord.[21] However, Mjellem et al.[22] showed that the activation of 5-HT_{1A} receptors inhibited whereas activation of 5-HT_2 receptors enhanced the behavior induced by NMDA or AMPA receptor activation. 5-$HT_{2A/2C}$ receptor-induced facilitation also occurred independently of NMDA and AMPA receptor activation.[23] The activation of 5-HT_3 receptors decreases NMDA-induced motoneuron depolarizations[24] and induces analgesic effect.[25] However, stimulation of 5-HT_3 receptors in the spinal cord can result in facilitation of pain transmission via increasing substance P release from the primary sensory afferents[26] or inhibition by increasing GABA release.[27] Serotonin decreased NMDA responses on dorsal horn synaptic transmission.[28] However, serotonin enhances L-glutamate-induced excitation of motoneurons in the spinal cord.[29] Therefore, serotonin might have either analgesic or algesic effect according to the receptors mainly act on and experimental conditions. Our previous study[3] showed analgesic effect of intrathecal serotonin on acute thermal and inflammatory acute and chronic pain. Spinal α_2-adrenergic and 5-HT_3 pathways share a common mechanism to reduce neuropathic pain after nerve injury.[15] We have shown intrathecal clonidine + epibatidine had synergistic analgesia in acute thermal and acute and chronic inflammatory pain.

We observed agitation and allodynia in one rat with 1/2ED50s of clonidine and serotonin. Our previous studies showed agitation and allodynia in one rat with serotonin 100 μg[3] and 2 rats with clonidine 0.3 μg[4], and another rat with clonidine 3 μg showed motor disturbance.[4] Therefore, behavioral side effects might decrease by the combination.

In conclusion, our study showed that for acute thermal and acute and chronic inflammatory pain, clonidine + serotonin might be better than clonidine + epibatidine when intrathecally administered.

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