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Original Article

The Effect of Clonidine Pretreatment on Epidural Resiniferatoxin in a Neuropathic Pain Rat Model

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Resiniferatoxin (RTX) is an ultrapotent synthetic TRPV1 (transient receptor potential vanilloid subtype 1) agonist with significant initial transient hyperalgesia followed by a prolonged analgesic effect in response to thermal stimulus. Using a rat model of neuropathic pain, we evaluated the effect of pretreatment with clonidine—which has been shown to relieve intradermal capsaicin-induced hyperalgesia—on the initial hyperalgesic response and the thermal analgesic property of RTX. Thirty-six male rats were divided into 6 treatment groups (n = 6 each): RTX 500 ng, RTX 1 μg, clonidine 20 μg (Cl), Cl+RTX 500 ng, Cl+RTX 1 μg, or normal saline 20 μL (control). We evaluated the short-term (180 min) and long-term (20 days) analgesic effects of RTX after thermal stimulation and mechanical stimulation. RTX had significant initial transient hyperalgesia followed by a prolonged analgesic effect in response to the thermal stimulus, but the RTX 500 ng and RTX 1 μg groups showed no initial short-term thermal hyperalgesic responses when pretreated with clonidine. The Cl+RTX 1 μg rats' behavior scores indicated that they were more calm and comfortable compared to the RTX 1 μg rats. Even though we cannot precisely confirm that pretreatment with clonidine potentiates or adds to the analgesic effect of RTX, clonidine pretreatment with epidural RTX eliminated the initial RTX-associated hyperalgesic response and systemic toxicity in this neuropathic pain rat model.

Key words: clonidine, epidural administration, resiniferatoxin, spinal nerve ligation rat model, thermal hyperalgesia

The chemical resiniferatoxin (RTX), which induces the long-term desensitization of nociception, provides conduction analgesia via selective blocking of transient receptor potential vanilloid subtype 1 (TRPV1), which is expressed by A-δ and C-fiber sensory neurons [1–3]. However, the use of RTX is controversial because its neurotoxicity and margin of safety have not been addressed adequately [4]. Upon an initial encounter with nociceptive fibers in rats, RTX induced a robust hyperalgesia response due to its

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ultrapotent capsaicin-like property [1]. Although this hyperalgesic response was transient, it appears to have a significant impact on the safety and efficacy of the RTX use [4].

In a previous study with rats, we observed prolonged thermal analgesia with 1-μg RTX without respiratory paralysis or sedation [5]. However, all of the RTX-treated rats showed distinct acute systemic toxicity (*e.g.*, irritation and somnolence) and a transient thermal hyperalgesic response to the initial administration of RTX [5]. The initial pain response has been a deterrent to widespread use of RTX [4].

In other studies, pretreatment with 0.5% bupivacaine prior to RTX reduced the pain, but pretreatment with 2% lidocaine was much less effective [2, 6]. Brown *et al.* [3] administered general anesthesia to dogs with bone cancer up to 60 min before administering intrathecal RTX to inhibit the initial hyperalgesic response. However, neither general anesthesia nor the use of bupivacaine to induce a nonselective motor and sensory block appears to be a practical pretreatment option.

Intrathecal clonidine was shown to relieve intradermal capsaicin-induced hyperalgesia in human volunteers [7], and Eisenach *et al.* [8] demonstrated that the potency of epidural clonidine is similar to that of intrathecal clonidine in chronic neuropathic pain patients, although the reasons for the similar potencies are unclear. No pain or agitated behavior associated with perineural clonidine injection has been reported [9]. Moreover, clonidine has been known to be more potent when given neuraxially than when given systemically [10].

The primary goal of the present study was to determine whether an epidural clonidine injection can reduce the initial thermal hyperalgesic response to RTX. The secondary goal of the study was to assess the effect of clonidine pretreatment on the long-term analgesic effect of RTX in a rat model of neuropathic pain.

Methods

Rat model of neuropathic pain. The experimental procedures were approved by our institution's Animal Care Committee. Male Sprague-Dawley rats purchased from Orient Bio (Seongnam, Korea) were housed in clear plastic cages with sawdust bedding at room temperature (20–22℃) and humidity (55–60%) under a 12-h light/dark cycle. The animals were acclimatized for at least 3 days prior to the study.

Seven-wk-old rats weighing 230–250 g were anesthetized with 2% isoflurane/O_2 via a loose-fitting mask. The spinal nerve ligation model was created with a cutting method [11]. For each animal, a 3-cm paramedian incision was made in the left L4-sacral area, and a bundle of paraspinal muscles was removed to visualize the left L6 transverse process. Using small scissors, we removed the left L6 transverse process completely and exposed the L4–L5 spinal nerves. After the L4 spinal nerve was separated, the L5 spinal nerve was cut and spread laterally. The fascia and skin were closed using sutures, and the animals were allowed to recover for 10 days prior to the epidural catheterization.

Animals that exhibited tactile hypersensitivity to von Frey filaments on the 10th day post-spinal nerve ligation were included in the treatment experiments. Rats that showed a motor deficiency or failed to exhibit tactile hypersensitivity were excluded from further testing.

Epidural catheterization. Rats were anesthetized using the technique described above. After sterile preparation of the skin, a 3-cm midline incision was made at the L1–L2 level. The superficial fascia and muscles were dissected, and the L1 and L2 spinous processes were exposed. Holding the L2 spinous process using tooth forceps, we removed the L1 spinous process, and the surrounding muscles were carefully separated until the ligamentum flavum was exposed. A small hole was made with a blunted 26-gauge needle at the center of the ligamentum flavum, and a PE-10 catheter (Becton Dickinson and Company, Franklin Lakes, NJ, USA) was advanced approx. 3 cm caudally until the tip reached the L4–L5 interspace [12]. The total tube length was 17 cm, and the dead space was 12 μL. Polyethylene glue (a cyanoacrylate adhesive; Aron Alpha, Toagosei, Tokyo, Japan) was applied around the catheter entry site to prevent drug leakage. The catheter was tunneled subcutaneously near the neck, and 0.1 mL of 2% lidocaine was injected as a test dose. Any rats for which an accidental intrathecal or intravenous injection of test agents led to sudden death were not included in the experiment [12]. After the correct epidural catheter placement was confirmed, the fascia

and skin were closed with sutures. If a rat exhibited a limp or spinal deformity during the 2-day observation period, it was excluded from the study.

To confirm that the injected agents reached the L4–L5 level, we administered 20 μL of Evans blue (Sigma-Aldrich, St. Louis, MO, USA) to 6 rats through the epidural catheter and sacrificed them by transcardial perfusion with 0.1 M phosphate buffer. In another 6 rats, 20 μL of Evans blue was injected twice, 20 min apart, and we visually confirmed that the injections also reached the L4–L5 epidural level, although 2 injections resulted in a wider distribution than a single injection.

Drugs and epidural administration. RTX 1 mg (Sigma-Aldrich) was dissolved in 95% ethanol (1 mL) to a concentration of 1 μg/μL and stored at −20℃. It was diluted using 0.9% normal saline to the required concentration [1, 13]. Clonidine hydrochloride (1 mg/mL) with no preservatives (lot 807126) was obtained from the Duke Compounding Pharmacy at Duke University Medical Center (Durham, NC, USA).

Thirty-six epidurally catheterized rats (weighing 280–330 g at that point) were prepared for the experiment. We administered the drugs 2 days after catheterization [14]. The rats were randomly divided into 6 treatment groups (n = 6 each): control (normal saline 20 μL), RTX 500 ng, RTX 1 μg, clonidine 20 μg (Cl), Cl+RTX 500 ng, and Cl+RTX 1 μg. The injection volume was 20 μL for each concentration.

Our decision to use a clonidine concentration of 20 μg was based in part on previous studies that reported that 20 μg was an optimal analgesic effect for intrathecal clonidine [15, 16]. In our preliminary study with the spinal nerve ligation model, rats were given either 20 μg or 40 μg clonidine epidurally (6 rats per group). The animals in the latter group were so sedated that they could not stand for about 30 min afterward, and the thermal thresholds were not superior to those for the clonidine 20 μg group (data not shown). For these reasons, we chose the clonidine concentration of 20 μg for our study.

We chose the RTX concentrations of 500 ng and 1 μg based on the results we obtained in our previous study [5] in which transient initial hyperalgesia and significant sedation were observed at doses ≥ 2 μg, whereas only initial thermal hyperalgesic responses were elicited at 500 ng and 1 μg. The efficacy of clonidine pretreatment was more prominent with RTX 1 μg than with RTX 500 ng.

The choice of a 20-min interval between the clonidine administration and RTX administration was based on our preliminary clonidine study. Following the epidural administration of clonidine 20 μg, the onset of analgesia in response to mechanical stimulation occurred slowly over the first 60 min, whereas the onset of analgesia in response to thermal stimulation occurred within the first 15 min. Hence, we felt that a 20-min interval would be sufficient to inhibit the initial RTX-associated hyperalgesic response to thermal stimulation.

After anesthetizing the rat with 2% isoflurane/O_2, we injected RTX or clonidine for 15–20 sec via an epidural catheter using a microinjector syringe (Hamilton Co. Reno, NV, USA). For the rats that received both clonidine and RTX, clonidine was injected first and RTX was injected 20 min later.

Behavioral tests. Mechanical stimulation and the thermal test were performed by the same researcher, who was blinded to the drugs injected. The rats' responses to a graded mechanical stimulus with von Frey filaments (Stoelting, Wood Dale, IL, USA) were measured. Each rat was placed under a transparent plastic dome on a metal mesh floor for 20 min. A von Frey filament was applied to the mid-plantar surface of the left hind paw for 2–3 sec. The 50% withdrawal threshold was determined using the up-down method [17] starting with a 2.0 g (19.608 mN) filament. A rapid foot lift upon filament contact was regarded as a withdrawal response. Interpolation of the 50% threshold was carried out according to the Dixon method [18].

To assess thermal pain sensitivity, we used a modified Hargreaves test [19] with a plantar test device (7,371; Ugo Basile, Comerio, Italy). Each rat was allowed to move freely for 20 min on a glass floor within an open-top transparent plastic box. The surface of the glass was maintained at 28–30℃ using a heating pad and an electric stove [11]. A mobile radiant heat source (infrared radiant intensity: 60) was applied through the glass platform to the plantar surface of the rat's left hind paw [20]. To prevent thermal injury, the exposure cutoff time was 15 sec [20]. No visible tissue damage was observed under these conditions. The average of 3 consecutive measurements was documented.

Behavioral tests were performed immediately before the spinal nerve ligation and before the initial epidural drug administration of clonidine or RTX, and subsequently at 15-min intervals for 180 min and then at 1, 3, 5, 7, 10, 15, and 20 days. At the same time, we observed the sedation status and behaviors of the rats of all groups. The same researcher who performed the mechanical and thermal tests examined the rats' behaviors, and he was blinded to the drugs injected. We did this experiment using rats that were reared by hand, and we always petted the rats for about 10 min before starting the experiment. After one researcher administered RTX to the rats, the other researcher observed the rats' behaviors continuously and recorded them. The sedative appearance of the rats were recorded according to Kawamata's sedation score [21].

Statistical analysis. The statistical analyses were performed using SigmaPlot for Windows version 12.0 (2011; Systat Software, San Jose, CA, USA). We used the Kruskal-Wallis one-way analysis of variance (ANOVA) by ranks to compare the responses to mechanical and thermal stimuli among the 6 treatment groups. For multiple comparisons after each analysis, the Tukey test was used. The data are expressed as means with standard deviations. *P*-values < 0.001 were considered significant. The significance of differences in the rats' responses to mechanical and thermal stimuli was determined for comparisons between the control group and other groups and between the RTX-only and Cl-RTX groups.

Results

Short-term responses to mechanical and thermal stimulation. The thresholds for the post-RTX withdrawal of the left hind paw in response to mechanical stimuli for the initial 180 min are shown in Fig. 1A. The Cl group threshold gradually increased for the first 60 min and remained steady thereafter, and was significantly higher than the corresponding thresholds for the control group ($p < 0.001$). The RTX 1 μg, Cl+RTX 500 ng, and Cl+RTX 1 μg groups showed almost immediate increases in the withdrawal threshold, which were maintained for 180 min; the results for these treatment groups were, for the most part, significantly higher than the results for the control group ($p < 0.001$) as well as the RTX 500 ng group ($p < 0.001$). There were no significant differences among the RTX 1 μg, Cl+RTX 500 ng, and Cl+RTX 1 μg groups. The increased withdrawal threshold for the RTX 500 ng group was not significantly different from the withdrawal threshold for the control group. The latencies of the rats' withdrawal from the thermal stimuli for the initial 180 min are shown in Fig. 1B. The Cl group showed a significant increase in withdrawal latency compared to the control group during the first 15 min after the injection ($p < 0.001$), with the increased withdrawal latency remaining steady throughout the 180 min. For the RTX 500 ng and RTX 1 μg groups, the initial latency was numerically lower than that of the control group during the first 60 min, even though there were no significant differences compared to the control group. Thereafter, the latency of the RTX 1 μg group increased substantially and plateaued, whereas the latency of the RTX 500 ng group increased at a much slower rate. We thus named the first 60-min. period after the 'post' state the hyperalgesic period and the time after this 60-min. period the analgesic period.

Among the RTX-treated rats (at all RTX concentrations), the withdrawal latency increased much more rapidly in the rats that were pretreated with clonidine than in those that were not pretreated. The withdrawal latency did not differ significantly between the RTX 1 μg and Cl+RTX 1 μg groups except for the first 60 min.

We observed and summarized the degree of sedation and behavior pattern of the rats in all groups in Table 1. The rats of the Cl group sat quietly, sometimes standing or grooming but with no spontaneous movement; for 30 min they moved when touched before returning to the pre-drug state over time. The rats in the RTX 500 ng and Cl+RTX 500 ng groups exhibited no abnormal behaviors. There were differences in behavior between the RTX 1 μg group and the Cl+RTX 1 μg group. Of the 6 rats in the RTX 1 μg group, 4 were aroused spasmodically and presented with very irritable behavior such as shoveling their feces; 5 scratched the metal mesh floor nervously, and 2 vocalized when the examiner touched them. Such behaviors persisted for up to 60–90 min. In contrast, the rats in the Cl+RTX 1 μg group did not exhibit irritable behaviors and remained calm and comfortable. The sedation status gradually went back to the initial state over time, and there were no motor

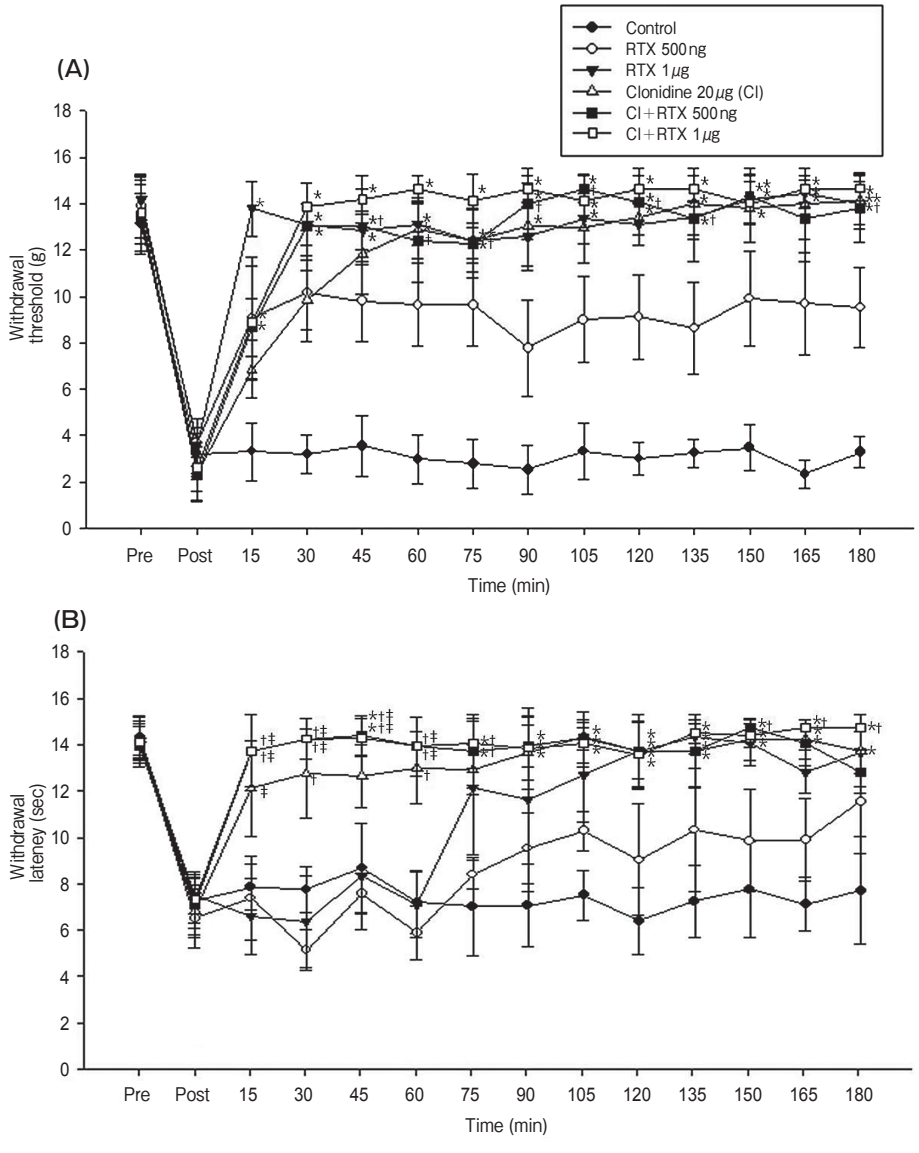

Fig. 1 Time course of the rats' left hind paw withdrawal threshold (**A**) and latency (**B**) in response to mechanical and thermal stimulation during the first 180 min after an epidural administration of clonidine (Cl), resiniferatoxin (RTX), or both. Rats were treated epidurally with normal saline (control group) (●), RTX 500 ng (○), RTX 1 μg (▼), clonidine 20 μg (Cl) (△), Cl+RTX 500 ng (■), or Cl+RTX 1 μg (□). (**A**) The withdrawal thresholds, determined at 15-min intervals, increased significantly in the Cl, RTX 1 μg, Cl+RTX 500 ng, and Cl +RTX 1 μg groups. (**B**) During the first 60 min, the RTX 500 ng and RTX 1 μg groups had an initial hyperalgesic response; rats that were pretreated with clonidine before receiving those doses of RTX did not have an hyperalgesic response. $^{*}P < 0.001$ vs. the control group, $^{\dagger}P < 0.001$ vs. the RTX 500 ng group, $^{\ddagger}P < 0.001$ vs. the RTX 1 μg group. All data are means ± SD (6 rats per group). Pre, before the spinal nerve ligation; Post, before the epidural drug injection.

impairments or autotomic appearances in any of the groups. The rats in all groups did not show any sedation or abnormal behaviors from days 1 to 20.

Long-term responses to mechanical and thermal stimulation. The post-RTX withdrawal thresholds of the left hind paw in response to mechanical stimuli over 20 days are shown in Fig. 2A. In the Cl+RTX 500 ng and Cl+RTX 1 μg groups, the withdrawal thresholds were initially substantially higher than in the corresponding groups without clonidine

Table 1 The sedative and agitated behaviors of the rats

Group	Agitated behavior	Sedation score[a]
Control	Normal behaviors (6/6)	0 (6/6)[b]
Clonidine 20 μg (Cl)	Quiet and comfortable appearance	1 or 2 for approx. 30min
RTX 500 ng	Normal behaviors, but an irritable appearance with touch	0 (6/6)
RTX 1 μg	Shoveling feces (4/6), scratching the metal mesh floor nervously (5/6), vocalization (2/6) for 60–90 min	Dozing off briefly and aroused spasmodically with very irritable behaviors (6/6)[c]
Cl+RTX 500 ng	Normal behaviors, no irritable appearance	1 or 2 for approx. 30 min
Cl+RTX 1 μg	Normal behaviors, no irritable appearance, very calm and comfortable	1 or 2 for 60–90 min

[a]Intensity of sedation using a modification of the scale proposed by Kawamata *et al*. [21].
0: normal behavior, alert to the environment, standing or grooming
1: sitting quietly, sometimes standing or grooming
2: sitting quietly, no spontaneous movement, but moved when touched
3: no spontaneous movement, moved when touched but flattened itself within several seconds
4: no spontaneous movement, did not move when touched
5: loss of righting reflex, unresponsive
[b]The number of animals that showed the occurrence out of the 6 rats in the group.
[c]The rats of the RTX 1 μg group also showed a sedative appearance, but all were startled again and again with simultaneous agitated behaviors compared to the rats of the clonidine group, so we did not apply the same sedative score rule in the RTX 1 μg group.

pretreatment. In the Cl+RTX 1 μg group, the threshold was significantly higher than that in the control group at days 1 and 3 ($p < 0.001$), whereas the threshold difference in the Cl+RTX 500 ng group was significantly higher than in the RTX 500 ng group at day 3 ($p < 0.001$). The withdrawal threshold for each group returned to the baseline by post-drug day 10. The Cl only group failed to show a sustained analgesic effect in response to mechanical stimulation, even though the withdrawal threshold had increased compared to the pre-drug injection level during the initial 180 min after the drug administration.

In the long-term study (Fig. 2B), the withdrawal latency in the Cl+RTX 1 μg group was significantly higher than that in the control group throughout the entire 20 days of observation, whereas the withdrawal latency in the RTX 1 μg group remained higher for 10 days; there were no significant differences between the RTX 1 μg and Cl+RTX 1 μg groups. In the RTX 500 ng and Cl+RTX 500 ng groups, the withdrawal latency was significantly increased for 3 days post-drug injection compared to the pre-drug injection, and it decreased thereafter to the pre-drug injection level. The Cl-alone group did not show a sustained analgesic effect in response to thermal stimulation, even though the withdrawal latency was increased for the initial 180 min after drug administration.

Discussion

The results of this study demonstrated that pretreatment with clonidine mitigated the initial thermal hyperalgesic response to RTX in a rat model of neuropathic pain. TRPV1 receptors play an important role in the development of allodynia and hyperalgesia following injury and the ensuing inflammatory conditions. RTX has an analgesic effect via TRPV1 binding and desensitizes only sensory neurons [1–3].

Sensory nerve endings release a variety of proinflammatory neuropeptides in neuroinflammation, such as calcitonin gene-related peptide, substance P, galanin, cholecystokinin, and somatostatin [4]. RTX has been demonstrated to reduce neuropathic pain by upregulating galanin message-associated protein, vasoactive intestinal polypeptide, neuropeptide Y, or nitric oxide synthase or by depleting calcitonin gene-related peptide or substance P from the affected neurons [4,

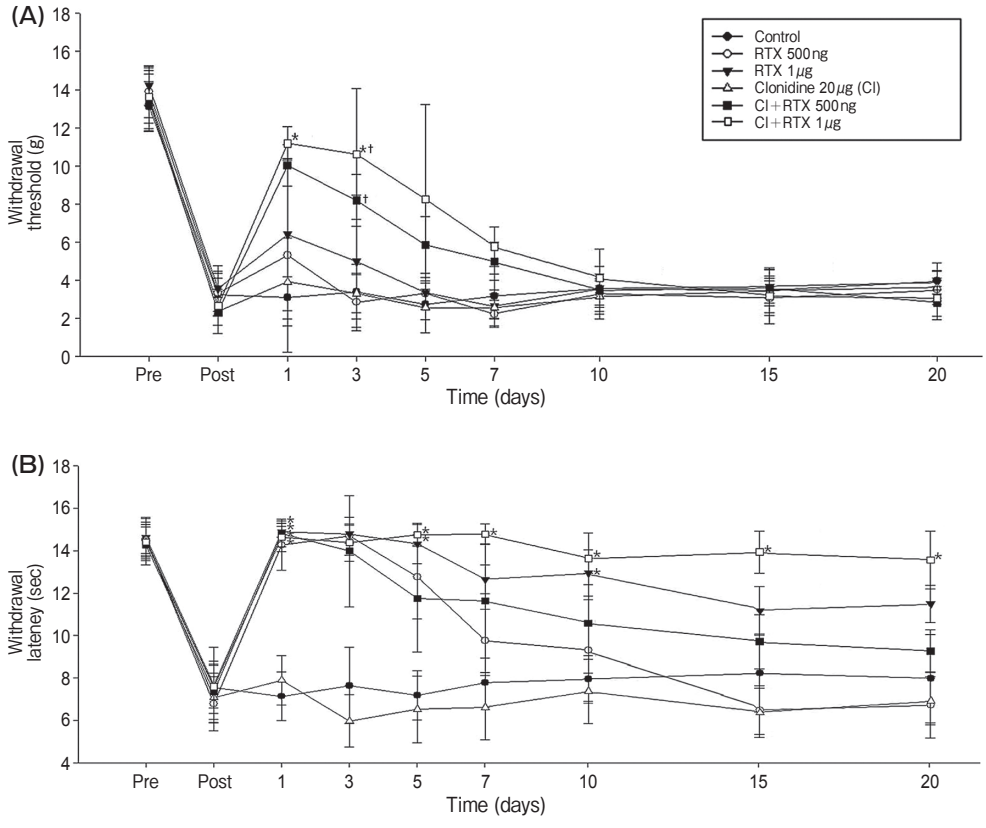

Fig. 2 Time course of the rats' left hind paw withdrawal threshold (**A**) and latency (**B**) in response to mechanical and thermal stimulation during the first 20 days after the epidural administration of clonidine, RTX, or both. Rats were treated epidurally with normal saline (control group) (●), RTX 500 ng (○), RTX 1 μg (▼), clonidine 20 μg (Cl) (△), Cl+RTX 500 ng (■), or Cl+RTX 1 μg (□). (**A**) The withdrawal thresholds increased significantly more in the clonidine-RTX groups than in the RTX-only groups, and the increased withdrawal threshold in the Cl+RTX 1 μg group was sustained for 3 days. (**B**) The withdrawal latency remained increased in the Cl+RTX 1 μg group for 20 days. $^{*}P < 0.001$ vs. the control group, $^{\dagger}P < 0.001$ vs. the RTX 500 ng group. All data are means ± SD (6 rats per group). Pre, before the spinal nerve ligation; Post, before the epidural drug injection.

22–24].

RTX produces an initial depolarization of primary sensory neurons, including TRPV1 receptors, causing pungency and irritation, followed by long-lasting desensitization of the nerve [1–3, 6]. Previous studies attributed the hyperalgesia initially induced by RTX to calcium accumulation within neurons, which in turn causes calcium cytotoxicity [3, 25]. Bupivacaine has been injected prophylactically in attempts to mitigate the initial excitatory responses to RTX, with limited success [2, 6]. Conduction blocks with local anesthetics have produced only short-term analgesia with nonselective motor or sensory dysfunction [2, 6].

An epidural RTX-induced thermal hyperalgesic response was shown to precede the analgesic effect [1, 5]. In the present study, even though the decrease in thermal latencies in the RTX-treated groups was not significant compared to the control group during the hyperalgesic period (first 60 min. after the post-state), dose-dependent effects of RTX were clearly shown during the analgesic period. We suspect that RTX-induced hyperalgesia might be buried in the already established thermal hyperalgesia of the neuropathic pain rat model, and this idea is consistent with the results of our precious study [5].

The unique properties of clonidine are well known. Intrathecal clonidine results in thermal and mechanical antinociception in a rat model of neuropathic pain [15, 21] and has an additive analgesic effect with local anesthetics and opioids [10]. Eisenach *et al.* [8]

reported that epidural or intrathecal clonidine alleviated intradermal capsaicin-induced allodynia in healthy volunteers. α_2-Adrenoceptor agonists such as clonidine increase the potency and efficacy of analgesia by concomitantly changing excitatory and inhibitory receptors in afferent neurons following peripheral nerve injury [10, 26].

In the present study, pretreatment with clonidine eliminated the early hyperalgesic response to RTX while improving the benefit of RTX by further increasing the withdrawal threshold to mechanical stimulation and sustaining the duration of the increased thermal withdrawal latency during short-term responses (180-min, Fig. 1A, B). Our findings confirmed the previous reports that the effect of clonidine is more prominent in the neuropathic pain state than under non-neuropathic conditions [7, 8, 10, 26–28]. When we observed the long-term analgesic effect of clonidine on RTX, the mechanical analgesic effect was very short (less than 3 days), and the difference in the thermal analgesic effect was not significant between the RTX group and clonidine-RTX group (Fig. 2). We were thus unable to observe the additive or potentiated effect of clonidine on RTX, which is a limitation of this study.

In contrast to our present findings, Chen *et al.* [16] observed that clonidine significantly potentiated the effect of intrathecal RTX, possibly due to reduced desensitization of α_2-adrenoceptors in the spinal cord. Ma *et al.* [26] reported that TRPV1 and α_{2C}-adrenoceptors, and their co-expression were up-regulated in injured dorsal root ganglion cell bodies after nerve ligation, and that the proportion of clonidine-inhibited cells that responded to capsaicin was increased by fivefold after nerve injury. Our study lacked detailed histological findings, and thus additional studies including immunostaining for TRPV1 receptor and α_2-adrenoreceptor are necessary to further investigate the potentiation of RTX effects by clonidine and to analyze any neurotoxic effects of clonidine-RTX combinations [29].

Clonidine-induced sedation is attributed to clonidine's rapid systemic absorption and redistribution to the central nervous system in a dose-dependent manner irrespective of the route of administration [7, 10]. Whether the rapid onset of the α_2-agonist-related analgesic property can be distinguished from the sedative effect is not clear, but it is known that clonidine eliminates the initial hyperalgesic response to RTX. In a typical clinical scenario, clonidine alone does not induce profound respiratory depression, and the level of sedation has been shown to be dose-dependent [10, 30]. Rauck *et al.* [27] reported similar degrees of systemic absorption and central distribution after cervical, thoracic, and lumbar epidural injections of clonidine, with minimal dispersion along the neuraxis.

Because the systemic absorption of clonidine after spinal administration is rapid, the onset of side effects from epidural clonidine occurs early [10, 28]. With the limited clonidine dose of 20 μg, we did not observe any significant side effects, abnormal behaviors, or deaths during the study period. We did not examine the potential side effects of hypotension, bradycardia, or respiratory difficulty.

In conclusion, RTX provided significant initial transient hyperalgesia followed by a prolonged analgesic effect in response to a thermal stimulus in a rat model of neuropathic pain. Although we cannot precisely confirm that pretreatment with clonidine potentiates or adds to the analgesic effect of RTX, we found that clonidine pretreatment with epidural RTX eliminated the initial RTX-associated hyperalgesic response and systemic toxicity in the neuropathic pain rat model used here. This finding contributes to the potential clinical uses of RTX in the future.

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