

Original Article

Suppression of complete Freund's adjuvant-induced adjuvant arthritis by cobratoxin

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Aim: Cobratoxin (CTX), the long-chain α -neurotoxin from *Thailand cobra* venom, has been demonstrated to have analgesic action in rodent pain models. The present study evaluated the anti-inflammatory and anti-nociceptive effects of CTX on adjuvant arthritis (AA) in rats.

Methods: Arthritis was induced by injection of complete Freund's adjuvant (CFA) in rats. Paw swelling and hyperalgesia of AA rats were measured at various times after CFA administration. Tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), interleukin-2 (IL-2) and interleukin-10 (IL-10) levels in serum were determined with ELISA. Histopathological changes in synoviocytes were examined under a microscope. Involvement of the cholinergic system in the effects of CTX was examined by pretreatment of animals with the α_7 nicotinic receptor (α_7 -nAChR) antagonist methyllycaconitine (MLA).

Results: CFA induced marked paw swelling and reduced thresholds of mechanical and cold-induced paw withdrawal. The levels of TNF- α , IL-1 and IL-2 in the serum of AA rats were increased, whereas the level of IL-10 was decreased. Histopathological examination of synoviocytes showed pronounced inflammation and accumulation of collagen. The administration of CTX (17.0 μ g/kg, ip) significantly reduced paw swelling and mechanical and thermal hyperalgesia. CTX also reduced the production of TNF- α , IL-1, and IL-2 but increased the production of IL-10 and altered pathohistological changes. The analgesic and anti-inflammatory efficacy of CTX was significantly reduced by MLA (3 mg/kg, sc).

Conclusion: These results indicate that CTX has a beneficial effect on CFA-induced arthritis by modulating the production of inflammatory cytokines. α_7 -nAChR appears to mediate the anti-nociceptive and anti-inflammatory actions of CTX.

Keywords: cobratoxin; adjuvant arthritis; anti-inflammatory; anti-nociception; α_7 -nicotinic acetylcholine receptor
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Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disorder that affects approximately 1% of the population worldwide. It is characterized by joint swelling, synovial membrane inflammation and cartilage destruction. The severity and disease progression of RA are governed by multiple factors including immune, genetic and environmental factors^[1, 2]. Multiple components of immunity and inflammation play a role in the onset and progression of the disease, including T and B lymphocytes, neutrophils, monocytes and

the vascular endothelium.

Several lines of recent evidence have suggested that proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1) play a pivotal role in the pathogenesis of RA because they are increased in the synovial tissue, synovial fluid and serum of RA patients. IL-1 and TNF- α contribute to synoviocyte self-proliferation and increase the production of tissue enzymes such as matrix metalloproteinases *via* chondrocytes and synovial cells, resulting in cartilage degradation^[3–5]. In the process of bone erosion, TNF- α triggers the production of other cytokines and endothelial adhesion molecules, stimulates collagenase and induces osteoclast differentiation^[6]. Furthermore, TNF- α exerts its arthritogenic potency through the induction of IL-1. Therefore, IL-1, IL-2, and TNF- α have been

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shown to be dominant players in the induction of inflammation and bone erosion^[7, 8]. In fact, anti-TNF- α antibodies and soluble TNF- α receptors have been proven to be effective in ameliorating RA^[9].

Snake venom, which contains small basic peptides, phospholipase A₂ (PLA₂), and PLA₂ homologs, has a significant effect on RA. Studies have shown that the *Bothrops atrox* venom peptide, batroxobin, caused a significant decrease in the fibrinogen level, whereas it had no effect on paw swelling^[10]. *Crotalus durissus terrificus* venom, which contains crotoxin as a major active component, administered subcutaneously before or after subplantar injection of carrageenan into the mouse right hind paw significantly inhibited edema and migration of polymorphonuclear cells to the peritoneal cavity^[11, 12]. Cobra venoms contain high levels of neurotoxins that target nicotinic acetylcholine receptors. Cobratoxin (CTX), a long-chain curaremimetic toxin from *Thailand cobra* venom, is composed of about 71 amino acids. The characteristic structure of CTX is a polypeptide chain organized in three major loops emerging like the fingers of a hand from a "palm" knotted together by the five disulfide bonds^[13, 14]. CTX has significant affinity for neuronal nicotinic acetylcholine receptor (nAChR) containing the $\alpha 7$ subunit^[15–17]. $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ -nAChR) are localized mainly presynaptically in the peripheral nervous system and also have high-affinity sites within the brain. It is also known that $\alpha 7$ -nAChR can conduct Ca²⁺ ions, thereby directly impacting neurotransmitter release^[18]. A previous study found that CTX exhibited a dose-dependent analgesic action in mice as determined by both the hot-plate and the acetic acid writhing tests. The analgesic effects of CTX were antagonized by atropine, but not naloxone. These results indicated that CTX produced potent opioid-independent analgesia^[19]. Recently, we also found that CTX exhibited a dose-dependent analgesic action in formalin-induced phase 1 and phase 2 pain responses and inhibited enhancement of c-Fos-positive cells in the spinal cord (Liu *et al*, manuscript submitted). However, its anti-inflammatory potential remains to be determined.

The present study, therefore, was designed to examine whether ip administration of CTX has anti-inflammatory effects in the rat adjuvant arthritis model, induced using complete Freund's adjuvant (CFA). In order to reveal the immunological influences of CTX, the serum levels of TNF- α , IL-1, IL-2, and IL-10 were measured and the histopathological changes in the joints of these AA rats were investigated. Furthermore, the involvement of the cholinergic system in the effect of CTX was examined by pretreatment of animals with methyllycaconitine (MLA), an $\alpha 7$ -nAChR antagonist.

Materials and methods

Animals

Male Sprague-Dawley rats weighing 180–220 g were purchased from the Experimental Animal Center of Soochow University. The animals were fed *ad libitum* and housed in a room with a controlled ambient temperature (22 \pm 2 °C), humidity (50% \pm 10%), and a 12-h light/dark cycle. Animals were acclimated to the housing conditions and handled for 3–4 days before experiments. All experiments were performed between 08:00 AM and 4:00 PM. All experimental procedures were conducted according to the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996). The experimental protocols were approved by the Local Committee on Animal Care and Use at Soochow University.

Reagents

CTX (99% pure), provided by ReceptoPharm (Plantation, FL, USA), was dissolved in saline and administered ip at doses of 8.5 and 17.0 μ g/kg. ELISA kits for TNF- α , IL-1, IL-2, and IL-10 were all purchased from Boster Biological Technology (Wuhan, China). CFA and methyllycaconitine (MLA) were purchased from Sigma.

Induction of arthritis

Male Sprague-Dawley rats were used in this study. Knee joint inflammation was induced by an intra-articular injection of 100 μ L CFA. As a control, 100 μ L of saline was injected. Procedures were performed under anesthesia with sodium pentobarbital (50 mg/kg, ip). The circumference of the knee joint was measured using a flexible tape meter.

CTX treatments

CTX (8.5, 17.0 μ g/kg) or saline (control) was administered ip. To study the effects of CTX on phase I (first inflammatory reaction) in AA rats, CTX (8.5, 17.0 μ g/kg) or saline was administered once daily for 3 d before administration of CFA. The last dose of CTX was administered 3 h before treatment with CFA. To study the effects of CTX or saline on the phase II response (secondary inflammatory reaction) in AA rats, CTX (8.5, 17.0 μ g/kg) was administered for 9 d (began at the 11th and ended at the 19th day) after the administration of CFA. MLA (3.0 mg/kg), dissolved in 0.9% saline and prepared freshly on the days of the experiment, was injected s.c. 2 h after the last injection of CTX. The dose of MLA was selected based on previous studies known to block $\alpha 7$ -nAChR^[20, 21].

Nociceptive behavioral tests

Mechanical paw withdrawal tests Rats were placed under a clear plastic cage on an elevated plastic mesh floor (1 cm² perforations) and were allowed to acclimate for 15 min before testing. Calibrated Von Frey monofilaments (North Coast Medical Co, USA) were used to deliver punctuated mechanical stimuli of varying intensities to the plantar surface of both hindpaws. Withdrawal response thresholds (g) for each hindpaw were determined using the up-down method described by Chaplan and colleagues^[22]. Each stimulus was applied for a duration of 1–2 s, with an inter-stimulus interval of 5 s. Care was taken to stimulate random locations on the plantar surface. Only robust and immediate withdrawal responses from the stimulus were counted. The mechanical force (in grams) applied by monofilament that produced a 50% withdrawal response was recorded as the threshold.

Cold plate test For assessment of hyperalgesia to cold stimulus in AA rats, a cold plate Analgesia Meter (homemade experimental device with temperature maintained at 2 °C) was used. Latency was defined as the length of time after the rats were placed on a cold plate (2 °C) before a hind paw began to shake. In all situations, the right paw injected with CFA reacted first.

Measurement of serum levels of TNF- α , IL-1, IL-2 and IL-10

The concentrations of TNF- α , IL-1, IL-2, and IL-10 in serum were determined with ELISA kits. On day 19 after CFA injection, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip). Blood was collected and stored at 4 °C for 30 min and then centrifuged at 3 000×g for 10 min. Supernatant was collected and stored at -20 °C until analysis. The levels of TNF- α , IL-1, IL-2, and IL-10 were determined using a commercially available enzyme immunoassay kits (Boster Biological Technology, Wuhan, China). Measurement was completed using an enzyme-linked immunosorbent assay with an absorbency maximum at 450 nm.

Assessment of synovial membrane pathology

On day 19 after CFA injection, rats were killed for dissection of their synovial membranes. Specimens were fixed for 6 h in 4% glutaraldehyde in PBS, decalcified and returned to 30% sucrose solution and submitted to routine paraffin embedding. Tissue sections were stained using hematoxylin and eosin (HE) or van Gieson's stain (VG).

Statistical analysis

Unless stated otherwise, data are expressed as means \pm SD and evaluated using ANOVA. Analysis of variance for repeated measurement was used where applicable. The *post hoc* test was also used with Student's Newman Keuls test when appropriate. $P < 0.05$ was considered statistically significant. Calculations were performed using the SPSS 10.0 statistical package.

Results

Effects of CTX on edema in the hind paw of adjuvant-induced arthritic rats

Figure 1 shows the measurements of circumference of knee joints after the administration of CFA. The CFA-injected paw remained swollen for more than 19 days. The curves of edema plotted against time could be divided into two phases. In the first phase, edema increased and reached a peak 3 days after CFA injection. Edema slowly subsided until the 9th day, when the paw began to swell again, and reached a second peak on the 11th day. Pre-treatment (3 d prior to CFA) or post-treatment (started on the 11th day after CFA) with CTX (17.0 μ g/kg, ip) significantly reduced CFA-induced paw edema compared with the group treated with CFA+saline.

For pre-treatments with CTX, edema was inhibited by 43% and 12% at 6 h (CTX 17.0 μ g/kg and 8.5 μ g/kg, respectively) and by 22% and 15% at 18 h (CTX 17.0 μ g/kg and 8.5 μ g/kg, respectively; Figure 1A). In phase II of CFA-induced adjuvant arthritis, CTX was administered on days 11–19 after the injection of CFA. There was a significant difference between the CTX- and saline-treated groups on the 11th and 19th day post-CFA. The most significant inhibition of CFA-induced edema (by 47% reduction) was observed on the 11th day after CFA administration (Figure 1B).

Effects of CTX on hyperalgesia in adjuvant-induced arthritic rats

CFA induced marked mechanical hyperalgesia, as evidenced by reduced nociceptive thresholds 11–19 days after CFA administration. This CFA-induced mechanical hyperalgesia was inhibited by post-treatment with CTX (17.0 μ g/kg, ip, once daily from the 11th to the 19th day after CFA injection; Figure 2A).

CFA also induced marked thermal hyperalgesia, as evidenced by reduced paw withdrawal latency, compared with the control group 11–19 days after administering CFA. CTX greatly increased paw withdrawal latency (Figure 2B). These

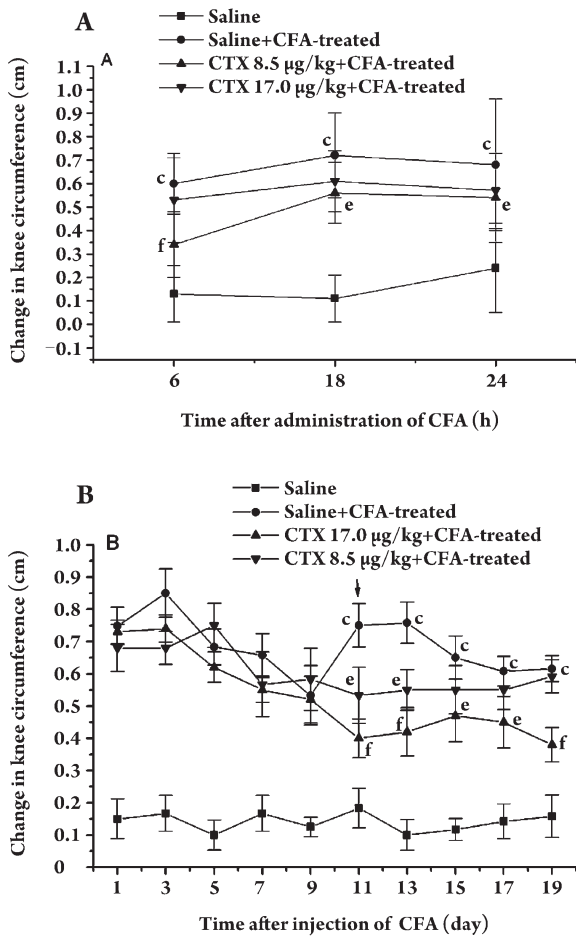


Figure 1. Effects of pre- and post-treatment with CTX on paw edema induced by CFA. Rats were treated with CTX (8.5, 17.0 µg/kg, ip) or saline once daily for 3 days before administration of CFA once daily for 9 days from the 11th to the 19th days after CFA injection. Paw edema was determined by measuring the circumference of the paw using a flexible tape measure 6, 18, and 24 h after CFA injection. Data represent the mean±SD (n=6). *P<0.01 compared with saline group; °P<0.05, †P<0.01 compared with saline+CFA-treated group. (A) pre-treatment with CTX once per day for 3 days before injection of CFA. (B) post-treatment with CTX for 9 days from the 11th day to the 19th day after CFA injection.

results indicate that CTX inhibits arthritic pain.

Effects of CTX treatment on levels of cytokines in AA rats

A significant increase in the concentrations of the proinflammatory cytokines IL-1, IL-2, and TNF-α occurred in the serum of arthritic animals on the 19th day after CFA treatment (Figures 3A–C). Post-treatment with CTX (17.0 µg/kg, ip, starting on the 11th and ending on the 19th day after CFA) had a marked inhibitory effect on the expression of

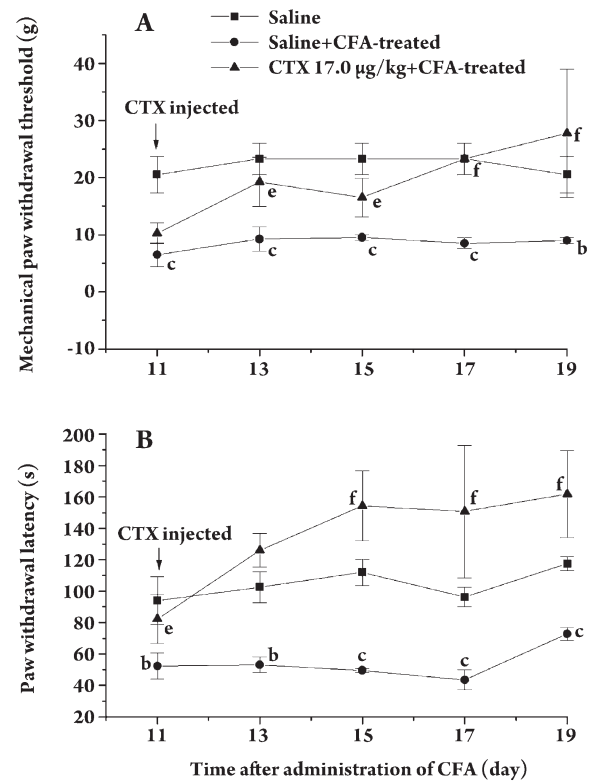


Figure 2. Effects of CTX on hyperalgesia in AA rats. Rats were treated with CTX (17.0 µg/kg, ip) once daily for 9 days from the 11th to the 19th day after CFA injection. Hyperalgesia was determined using Von Frey's Filaments test and a cold plate test. Data represent the mean±SD (n=6). *P<0.05, °P<0.01 compared to saline group; †P<0.05, ‡P<0.01 compared to saline+CFA-treated group. (A) Von Frey's Filaments test; (B) Cold plate test.

cytokines induced by CFA. However, serum IL-10 concentration decreased compared with that of saline control rats after CFA treatment. Post-treatment with CTX significantly reversed the CFA-induced decline in IL-10 (Figure 3D).

Effects of CTX on histopathology of synovium in AA rats

Histopathological analysis of the synovium of AA rats using HE staining showed clear inflammatory cell infiltration, edema and pannus formation. These abnormalities were significantly alleviated in AA rats after the administration of CTX (once daily from the 11th to the 19th day after CFA; Figures 4A–4C). Furthermore, VG staining showed marked hyperplastic cartilage, which was significantly inhibited by the administration of CTX (once daily from the 11th to the 19th day after CFA; Figures 4D–4F).

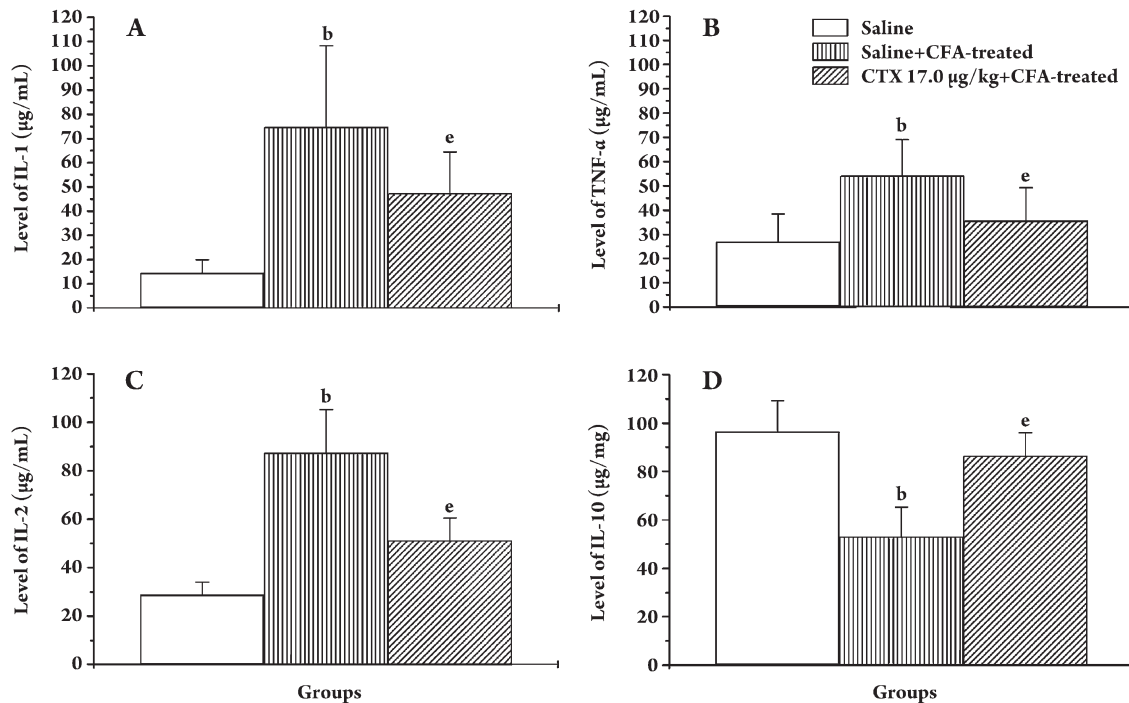


Figure 3. Effects of post-treatment with CTX on cytokine levels in the serum of AA rats. Rats were treated with CTX (17.0 μg/kg, ip) once daily for 9 days from the 11th to the 19th day after CFA injection. On day 19 after CFA injection, the rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), and blood was collected. The concentrations of TNF-α, IL-1, IL-2, and IL-10 in serum were determined with ELISA kits. Data represent the mean±SD ($n=6$). ^b $P<0.01$ compared with saline group; ^e $P<0.05$ compared with saline+CFA-treated group.

Effects of MLA on CTX's anti-inflammatory action in AA rats

To show that CTX's anti-inflammatory activity is mediated by the nicotinic cholinergic system, MLA (3.0 mg/kg, sc), an α_7 -nAChR antagonist, was administered 2 h after the injection of CTX, and CFA-induced paw edema was assessed 6–42 h after CFA administration. When used alone, MLA (3.0 mg/kg, sc) had no effect (11%) on CFA-induced paw edema, whereas CTX alone significantly inhibited CFA-induced paw edema in rats 42 h after CFA treatment (39% reduction). MLA antagonized the inhibitory effects of CTX on CFA-induced paw edema (Figures 5A and B). These results indicate that MLA could inhibit CTX's anti-inflammatory action.

Effects of MLA on CTX's anti-nociceptive effects in AA rats

Rats were treated with CTX (17.0 μg/kg, ip) or saline once daily for 3 days before administration of CFA. CFA induced marked mechanical hyperalgesia, as evidenced by reduced nociceptive thresholds compared with saline groups at 6, 24, and 42 h after administration of CFA. This CFA-

induced mechanical hyperalgesia was inhibited by CTX (17.0 μg/kg, ip). MLA (3.0 mg/kg) did not affect mechanical hyperalgesia induced by CFA, but it did robustly inhibit the effects of CTX's anti-nociceptive effect in rats at 6 h, 24 h, and 42 h after CFA administration (Figures 6A and 6B).

Discussion

AA has been used as a model of sub-chronic or chronic inflammation in rats and is of considerable relevance to the study of pathophysiological and pharmacological control of inflammatory processes, as well as for the evaluation of the analgesic potential or anti-inflammatory effects of drugs^[23,24]. AA elicits joint swelling, synovial membrane inflammation, and cartilage destruction. In addition, it is marked by increased sensitivity of the affected paw to pressure or thermal stimulus. The arthritis observed in rats is associated with a hyperalgesic phenomenon and spontaneous behaviors, such as protecting the affected paw and avoiding putting body weight on the paw. The hyperalgesia is more evident during the acute inflammatory phase^[25]. The present study demonstrates that CFA containing killed *M tuberculosis*

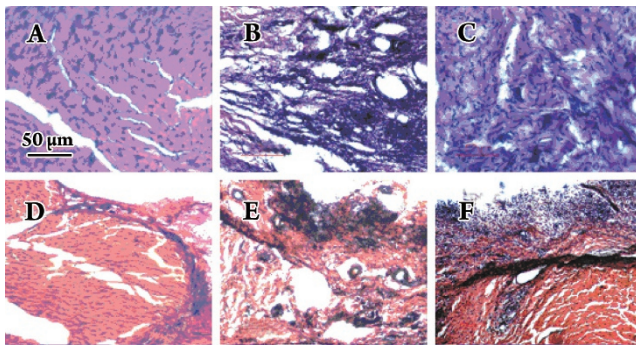


Figure 4. Effects of post-treatment with CTX on histological alterations of synovium. Rats were treated with CTX (17.0 $\mu\text{g}/\text{kg}$, ip) once daily for 9 days from the 11th to the 19th day after CFA injection. On day 19 after CFA injection, rats were killed and synovial membranes were removed. Tissue sections were stained with hematoxylin and eosin (HE) or van Gieson's stain (VG). (A) Saline group: normal articular cartilage and absence of infiltrate in the synovium (HE stain, $\times 400$); (B) Saline+CFA-treated group: marked infiltration of inflammatory cells and pannus formation (HE stain, $\times 100$); (C) CTX+CFA-treated group: minor infiltration of inflammatory cells (HE stain, $\times 100$); (D) Saline group: normal collagen (VG stain, $\times 100$); (E) Saline+CFA-treated group: hyperplastic collagen in the synovium (VG stain, $\times 100$); (F) CTX+CFA-treated group: decreased hyperplastic collagen (VG stain, $\times 100$).

induced AA in rats with joint swelling, hyperalgesia and synovial membrane inflammation. This effect of CFA is consistent with the results of other studies that have examined the action of CFA-induced AA in rats^[26]. Treatment of AA rats with CTX inhibited paw swelling, hyperalgesia, joint inflammation and, importantly, damage to the synovium, indicating that CTX might be an effective treatment for rheumatoid arthritis.

The pro-inflammatory cytokines TNF- α , IL-1, and IL-2 have been shown to play an important role in the pathophysiology of arthritis development in animal models and humans. It was reported that increased expression of inflammatory cytokines, including TNF- α and IL-1 β , was observed in the bone region of the knee joint or serum samples from human osteoarthritis or rheumatoid arthritic patients^[27]. TNF- α and IL-1 β enhance the proliferation of fibroblasts, stimulate the production of PGE₂^[28], and increase the expression of other cytokines and synthesis of collagen by synovial cells, contributing to cartilage and bone destruction^[29]. Thus, various strategies to block their activity are now being clinically applied and have been shown to be effective in the treatment of experimental arthritis^[30]. In the present study, the anti-inflammatory action of CTX is associated with significantly reduced IL-1 and TNF- α levels in the sera of AA rats.

It has been suggested that the anti-inflammatory cytokine

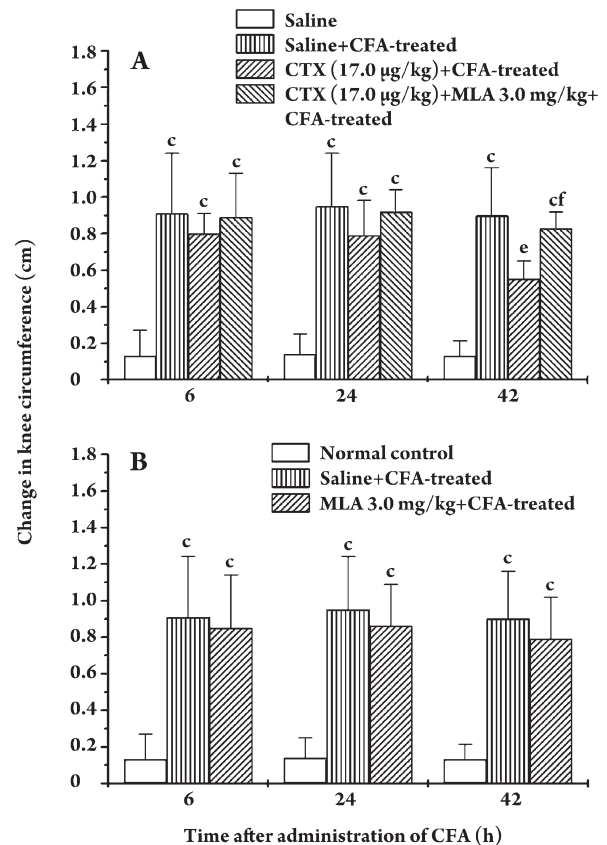


Figure 5. Effects of MLA on CTX's anti-inflammation effects (A) and CFA-induced paw edema (B) in AA rats. Rats were treated with CTX (17.0 $\mu\text{g}/\text{kg}$, ip) or saline once daily for 3 days before the administration of CFA. MLA (3.0 mg/kg) or saline was given 2 h after the injection of CTX. Paw edema was determined by measuring the circumference of the paw using a flexible tape measure 6, 24, and 42 h after CFA injection. Data represent the mean \pm SD of 6 rats. ^c $P < 0.01$ vs saline group; ^e $P < 0.05$, ^f $P < 0.01$ vs saline+CFA-treated group.

IL-10 is an important factor in resolving chronic inflammation. IL-10 inhibits the production of proinflammatory cytokines including IL-1, TNF- α , and IL-2^[31, 32]. IL-10 administration suppressed the progression of arthritis in animal models, and clinical improvement has been reported in rheumatoid patients treated with recombinant human IL-10^[33, 34]. In this report, consistent with previous studies, a decrease in IL-10 levels in the serum of AA rats was found^[35]. The administration of CTX markedly increased IL-10 levels. Although this increased production of IL-10 cannot be definitively attributed to CTX's anti-arthritis actions, it is clear that CTX shifts the balance of the cytokine parameters in the serum away from pro-inflammatory cytokines (TNF- α , IL-1, and IL-2) and toward the production of anti-inflammatory cytokines such as IL-10.

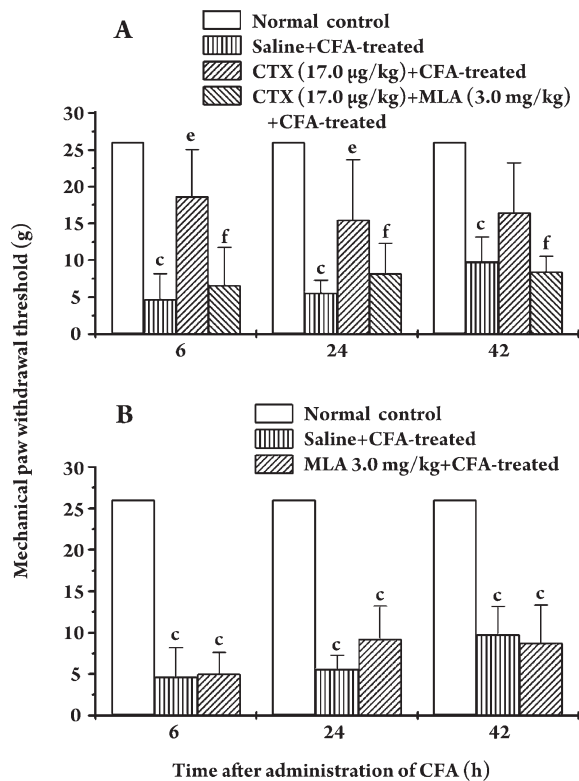


Figure 6. Effects of MLA on CTX's anti-nociceptive effects (A) and CFA-induced mechanical hyperalgesia (B) in AA rats. Rats were treated with CTX (17.0 µg/kg, ip) or saline once daily for 3 d before the administration of CFA. MLA (3.0 mg/kg), or saline was given 2 h after the injection of CTX. Paw withdrawal thresholds were measured using Von Frey's Filaments test at 6, 24, and 42 h after administration of CFA. Data represent the mean±SD of 6 rats. ^c*P*<0.01 vs saline group; ^e*P*<0.05, ^f*P*<0.01 vs saline+CFA-treated group.

A number of recent studies have indicated that the cholinergic anti-inflammatory pathway is an important regulator of cytokine-mediated damage in local and systemic experimental diseases such as arthritis and pancreatitis. Intracerebroventricular administration of muscarinic receptor agonists significantly decreased serum TNF- α levels, indicating that muscarinic brain networks regulate the cytokine-controlling function of the vagus nerve^[36]. Epibatidine, a potent agonist of nAChRs, has structural and functional characteristics similar to that of acetylcholine and nicotine and it attenuated increases in joint circumference and pain-related behaviors induced by injecting a mixture of kaolin and carrageenan into the knee joint. The effects of epibatidine were altered by mecamylamine, a nicotinic receptor antagonist^[37]. The administration of α_7 -nAChR agonists inhibits not only TNF- α but also IL-1, IL-6, IL-8, and high mobility group box1 (HMGB1)^[38]. Furthermore, a number of studies indicate that cholinergic signaling mediated by α_7 -nAChR has

been found in cytokine-producing cells. Cholinergic signals derived from vagus nerve stimulation inhibit the release of TNF, IL-1, HMGB1, and other cytokines by transducing cellular signals that inhibit the nuclear activity of NF- κ B^[38].

In contrast to the roles of nicotinic cholinergic receptors agonists discussed earlier, the present study indicates that CTX, which may be a nicotinic antagonist, also has a role in inflammatory relief^[19, 39]. As far as we know, no reports demonstrate that CTX interacts with any receptor other than AChR. In the present study, α_7 -nAChR antagonism was induced by MLA, which was ineffective in AA rats by itself. However, co-administration of MLA with CTX antagonized the analgesic and anti-inflammatory effects of CTX in AA rats. Previous studies have shown similar results with atropine, a muscarinic cholinergic receptor antagonist. In the hot-plate and acetic acid writhing tests, atropine antagonized the analgesic effects of CTX. We also recently found that atropine antagonized the analgesic action of CTX and canceled the inhibitory effect of CTX on formalin-evoked c-Fos expression in the spinal cord of formalin-evoked rat pain models (Liu *et al*, manuscript submitted). These results indicate that CTX produced potent analgesia or anti-inflammatory effects, which may be mediated by activation of acetylcholine receptors. Therefore, we presume that CTX might exert its activity through α_7 -nAChR. This possibility, however, needs further investigation.

In summary, the present study demonstrates that the ip administration of CTX, a long chain α -neurotoxin, has distal anti-inflammatory effects in addition to providing anti-nociception in the rat adjuvant arthritis model, possibly by inhibiting the release of cytokines including TNF- α and IL-1 and by preventing joint destruction, which is mediated by activation of the acetylcholine receptor. The positive effect of CTX in rats with AA due to the modulation of inflammatory cytokines and the ability to control pain may be beneficial to human subjects with rheumatoid arthritis.

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Author contributions

Zheng-hong QIN, Yan-li LIU designed research; Yan-li LIU, Hai-ming LIN, Rong ZOU, and Jun-chao WU performed research; Rong HAN, Laurence N RAYMOND, Paul F REID contributed new analytical tools and reagents; Yan-li LIU analyzed data; Yan-li LIU, Zheng-hong QIN, Paul F

REID wrote the paper.

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