

Trifluoro-Icaritin Ameliorates Complete Freund's Adjuvant-Induced Inflammatory Pain by $\alpha 7$ nAChR-Mediated Inhibition of HMGB1/NF- κ B Signaling in the Spinal Cord of Rats

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Abstract

Objectives: To explore whether Trifluoro-icaritin (ICTF) has anti-nociceptive effect on CFA-induced inflammatory pain and its potential mechanisms.

Methods: Intraperitoneal injection (0.3, 1.0, and 3.0 mg/kg, i.p.) of ICTF to complete Freund's adjuvant (CFA)-induced inflammatory pain rats once daily for 21 consecutive days. Pain-related behaviors were evaluated with paw withdrawal threshold (PWT), paw withdrawal latency (PWL), and CatWalk gait analysis. Hematoxylin eosin (HE) staining was applied to determine the morphological alterations in inflamed paw. Molecular docking was conducted to assess the possible targets for ICTF. Expression of pain-related signaling molecules in the spinal cord were detected using qRT-PCR, western blot assay, and immunofluorescence staining.

Results: This results showed that ICTF(3.0 mg/kg) effectively alleviated mechanical allodynia and thermal hyperalgesia but not 0.3 and 1.0 mg/kg in CFA rats. Both paw edema volume and paw tissue inflammatory response were obviously reduced by ICTF. Subsequently, we further observed that ICTF dramatically decreased the mRNA and protein levels of HMGB1, NF- κ B p65, and IL-1 β but markedly enhanced α 7nAChR and IL-10 expression in the spinal cord of CFA rats, and inhibited the co-expression of spinal α 7nAChR with IBA-1 in double immunofluorescence staining, along with suppressing the alterations of gait parameters induced by CFA. Moreover, Intrathecal injection (i.t.) of α 7nAChR antagonist alpha-bungarotoxin (α -Bgtx, 1.0 μ g/kg) not only reversed the anti-nociceptive effect of ICTF on pain hypersensitivity, but also inhibited the down-regulation of HMGB1, NF- κ B p65, and IL-1 β as well as the up-regulation of α 7nAChR and IL-10 protein expression induced by ICTF treatment.

Conclusion: Our results illustrate that ICTF enables to alleviate CFA-induced inflammatory pain through α 7nAChR-mediated inhibition of HMGB1/NF- κ B signaling pathway in the spinal cord of rats, suggesting that ICTF may be exploited as a potential painkiller against chronic inflammatory pain.

Background

Chronic inflammatory pain has been a major public health issue worldwide, which severely impacts patients' life quality and causes a great economic burden(1, 2). Chronic inflammation always enables to provoke persistent pain that characterized by hyperalgesia, allodynia, and spontaneous pain(3). During this process, several pro-inflammatory mediators, such as cytokines, chemokines, and nerve growth factors, are continuously released at the inflammation site, which promotes the sensitization of peripheral nociceptors(4), and ultimately elicits pain hypersensitivity. Since the pathophysiology of chronic inflammatory pain is relatively complicated, including peripheral and central mechanisms, and its pathogenesis remain poorly understood. Thereby the clinical management for chronic inflammatory pain still confronts extremely challenges(5). Neuroinflammation has been documented to participate in the pathogenesis of chronic inflammatory pain(6). More importantly, under inflammatory pain states, neuroinflammation involves the activation of microglia, the release of pro-inflammatory mediators and

the overexpression of pain-related signaling molecules(7). Thus, targeting these signaling molecules may be potential therapeutic strategies against chronic inflammatory pain(5).

Nowadays, cholinergic anti-inflammatory pathway has attracted much attention in the study of chronic inflammatory pain(8). The pathophysiological mechanism underlying inflammatory pain in which nervous system interacts with immune system to provoke systemic inflammation(9). There is convincing evidence of a critical importance of $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) in mediating cholinergic anti-inflammatory pathway(10). It is known that $\alpha 7$ nAChR, largely expressed in immune cells, neuron, microglia, and astrocyte, exhibits an anti-inflammatory effect during immune response(11–13). Further, $\alpha 7$ nAChR is identified to play an important role in modulation of chronic inflammatory pain(14). Evidence showed that high mobility group box 1 (HMGB1) is a highly conserved and ubiquitous nuclear protein that released into the extracellular environment as a key pro-inflammatory mediator(15). Within the process of inflammatory response, HMGB1 has been verified to be involved in neuroinflammation. However, $\alpha 7$ nAChR enables to reduce the inflammatory response by suppressing HMGB1 release(16). It is well established that complete Freund's adjuvant (CFA) induces inflammatory pain through triggering HMGB1, and resulting in pro-inflammatory cytokines (IL-1 β , TNF- α , and IL-6) release by activating NF- κ B signaling(17). Besides, it is reported that microglia which extremely existed in the spinal cord is an important natural immune cell in central nervous system(18) and microglia-mediated neuroinflammation is involved in the development and maintenance of inflammatory pain(19). Overall, these lines of evidence strongly supported that suppression of HMGB1/NF- κ B signaling pathway following activation of $\alpha 7$ nAChR may be a promising treatment for chronic inflammatory pain.

Currently, chronic inflammatory pain is still a serious clinical problem to be urgently solved, and available managements for chronic inflammatory pain including opioid analgesics and non-steroidal anti-inflammatory drugs (NSAID)(20), but these are often associated with clinical limitation owing to their larger side effects and unsatisfactory efficacy. Thus, it is very essential to develop novel and potent candidates for chronic inflammatory pain. Traditional Chinese medicine is a multi-target option that have profound anti-inflammatory effects on chronic pain with considerably fewer side effects(21). Evidence showed that icaritin (ICT) is a bioactive ingredient extracted from the dried stems and leaves of a genus of *Epimedium* plant and has anti-inflammatory and analgesic activities(22), which enables to reduce acetic acid-induced writhing times in a dose-dependent manner(23). Our previous studies have revealed that trifluoro-icaritin (ICTF), a derivative of ICT, exhibits a neuroprotective effect by reducing excitotoxicity and cerebral ischemia-reperfusion injury(24). Additionally, ICTF effectively promotes the repair of neural function through blocking excessive autophagy and Notch1 signal activation(25). Since neuroinflammation has been confirmed to be an important and inevitable pathophysiological process mediating peripheral nerve or tissue damage and dysfunction, including inflammatory pain, neuropathic pain, and cerebral ischemia-reperfusion injury(6). Thereby, whether ICTF possesses an anti-nociceptive effect on CFA-induced inflammatory pain need further investigation. Based on the fact that the activation of $\alpha 7$ nAChR mediates suppression of HMGB1/NF- κ B signaling pathway in chronic inflammatory pain(16). Accordingly, we hypothesized that ICTF may exert a potential inhibitory effect on CFA-induced

inflammatory pain via inhibiting HMGB1/NF- κ B signaling following α 7nAChR activation in the spinal cord of rats.

Therefore, based on the properties of ICTF, it may provide greater benefit than several conventional analgesics for chronic inflammatory pain relief, but its mechanisms underlying anti-inflammatory activity remains to be fully elucidated. Hence, in this study, α 7nAChR, HMGB1/NF- κ B signaling pathway and its downstream inflammatory cytokines (IL-1 β and IL-10) are regarded as several research highlights, and a rat model of CFA-induced inflammatory pain was utilized to investigate whether ICTF has anti-inflammatory, anti-swelling, and anti-nociceptive activities. To further explore whether the possible inhibitory effect of ICTF on CFA-induced inflammatory pain may through α 7nAChR-mediated inhibition of HMGB1/NF- κ B signaling pathway in the spinal cord of rats. Findings from this research will provide new therapeutic targets for ICTF treatment of chronic inflammatory pain.

Materials And Methods

Animals

Healthy male SD rats weighing 160–180 g (7 to 8 weeks old) were used in this study, which are provided by Hunan SLAC Laboratory Animal Co., Ltd. (Changsha, People's Republic of China). Rats were housed in a temperature-controlled (22°C-24°C) room with a 12/12-hour light/dark cycle, and they were kept five per cage with free access to food pellets and water. All animal experimental procedures strictly followed the guidelines of International Pain Research Association and have been approved by the Animal Protection and Use Committee of Gannan Medical University in P.R. China. The behaviors experiment were carried out in a quiet environment, and all tests were performed double-blindly. Each possible evaluation was performed to minimize pain to rats. A total of 164 rats were used in this research.

Reagents and drugs

Trifluoro-icaritin (C₂₁H₁₇F₃O₅) (Fig. 2A) was obtained from Shanghai Chenxiang Pharmaceutical Technology Co., Ltd. (No: CX190625001), with the purity of more than 98 %, the molecular weight of 406.3518896, and yellow powder. ICTF was dissolved in dimethyl sulfoxide (DMSO), and DMSO was purchased from Solarbio (No: d2650). α 7nAChR antagonist alpha-bungarotoxin (α -Bgtx, ab120542, Abcam, UK) was dissolved in PBS to the concentration of 1.0 μ g/kg. Complete Freund's adjuvant (CFA) was purchased from Sigma. Isoflurane was purchased from Jiangsu hengfengqiang Biotechnology Co., Ltd.

Experimental protocols

Animal experimental procedures for CFA-induced inflammatory pain were conducted in this study (Fig. 1). Firstly, in order to evaluate the possible effect of ICTF on pain hypersensitivity after injection of CFA, rats were randomly assigned to one of 6 groups: Ctrl, CFA, CFA + DMSO, CFA + ICTF (0.3 mg/kg), CFA + ICTF (1.0 mg/kg), and CFA + ICTF (3.0 mg/kg) (n = 6–10). Rats in The Ctrl group suffered injection of same

volume of normal saline (NS) to the left hind paw, while rats in the other groups received injection of 100 µl CFA to the left hind paw. Rats in the CFA + ICTF groups were treated with ICTF (i.p.) at doses of 0.3, 1.0, and 3.0 mg/kg on day 1 post-CFA injection and once daily (PM: 18:30) lasting for 14 days, respectively. Rats in the CFA + DMSO group were given with DMSO. The PWT and PWL at the ipsilateral hind paw of rats were measured following treatment of DMSO and different doses of ICTF on day 1 before and day 1, 3, 7, 10, and 14 post-CFA injection, respectively, and then statistical analysis was performed to determine the effective dose of ICTF.

Secondly, the effective dose of 3.0 mg/kg ICTF was selected for the next experiment, rats were randomly divided into 3 groups: Ctrl, CFA, and CFA + ICTF (3.0 mg/kg) group (n = 9–13), the assessment of the time-course effects of ICTF on the PWT, PWL, paw edema volume and CatWalk gait parameters were detected on day 1 before and day 1, 7, 14, and 21 post-CFA injection with once daily (PM: 18:30). Subsequently, to further investigate the potential mechanism underlying ICTF relieving CFA-induced inflammatory pain in the spinal cord. Rats from each group were underwent to dissect the L4-L6 segments of spinal cord and the left paw tissue of rats on day 21 post-CFA injection after behavioral experiments. These samples of spinal cord were obtained for detecting the levels of $\alpha 7nAChR$, HMGB1, NF- κB p65, IL-1 β , and IL-10 in both mRNA and protein by qRT-PCR and western blot assay. Additionally, these samples of the left paw tissue of rats were performed for HE staining.

Thirdly, in order to determine the possible effects of $\alpha 7nAChR$ antagonist α -Bgtx on ICTF producing the alleviation of pain hypersensitivity in CFA rats, rats were randomly assigned to one of 2 groups: CFA + ICTF + α -Bgtx and CFA + ICTF + PBS groups (n = 9–12). Intrathecal injection of 1.0 µg/kg α -Bgtx or PBS to CFA rats starting from day 14 lasting for 7 consecutive days following ICTF treatment with once daily (PM: 18:30), and the PWT and PWL were examined on day 21 post-CFA injection, respectively. Furthermore, rats in CFA + ICTF + α -Bgtx and CFA + ICTF + PBS groups were underwent to harvest the L4-L6 segments of spinal cord on day 21 post-CFA injection. The samples were collected for evaluating the protein expression of $\alpha 7nAChR$, HMGB1, NF- κB p65, IL-1 β , and IL-10 using western blot assay.

A rat model of CFA-induced inflammatory pain

Following rats were adapted to the surrounding for 5 days, a rat model of CFA-induced inflammatory pain was established according to the method described in our previous studies(26). In brief, rats were anesthetized by using isoflurane at a concentration of 2 % -3 %. One hundred microliter of CFA was injected into the rat plantar surface of left hind paw using a syringe with a 28-gauge needle. The control rats was performed by injection of same amount of normal saline (NS) into the same position of left hind paw. Rats then were kept at room temperature ($22 \pm 1^\circ C$) for recovery, and further experiments were performed on day 1 following CFA injection.

Assessment of mechanical allodynia

The sensitivity to mechanical stimulation was evaluated by measuring PWT as described in our previous study(27). Briefly, rats were firstly acclimatized in individual plastic enclosures (12 × 22 × 18 cm) on a metal mesh floor standing for 15 min before testing. The PWT was detected by application of a dynamic

plantar aesthesiometer (Ugo Basile, 37400-002, Italy), which comprises a force transduction fitted with a 0.5 mm diameter polypropylene rigid tip. A probe was used perpendicularly to the mid-plantar surface of the rat left hind paw with an increasing pressure, and spending 10 s from 0 to 50 g. To avoid of injure to rat, the cutoff pressure was set to be 50 g, the force that elicited the withdrawal response was automatically recorded to the nearest 0.1 g by the anesthesiometer. Three measurements were taken with an interval of 5 min. The mean PWT was obtained for each rat.

Measurement of thermal hyperalgesia

The sensitivity to thermal stimulation was assessed by detecting PWL of the rat left hind paw. This procedure was conducted as described in the previous report(28). Briefly, rats were acclimatized in individual plastic enclosures (12 × 22 × 18 cm) on a metal mesh stand for 15 min before testing. The thermal pain threshold was determined by application of a plantar tester (Ugo Basile, 37370-002, Italy). The radiant heat source was focused on the plantar surface of the left hind paw, in order to avoid rat paw tissue injury, the light intensity was reset to obtain a baseline latency of approximately 15 s. Three measurements were taken with an interval of 5 min. The mean PWL was obtained for every rat.

Evaluation of paw volume

A special instrument of plethysmometer (Ugo Basile, 37140, Italy) was applied in this experiment, the procedure was performed as previously described(29). In brief, the paw volume of rat was detected by adding 1L of 0.9 % sodium chloride solution and 1 ml of trelux into the measuring cup. The liquid level is between two red lines and calibrated with a probe, and then a marking line was drawn on the same position of the left and right ankles of rat. After rat was kept calm, the left hind paw of rat was put into the measuring cup. When the liquid level was coincided with the marking line, the pedal was stepped to record the measuring value, three measurements were taken to be the mean value for each rat. The right hind paw of rat was also detected using the same method.

Hematoxylin and Eosin (HE) staining

In this experiment, after establishment of a rat model of CFA-induced inflammatory pain, HE staining and inflammation scoring were performed for detecting the morphological changes in the inflamed paw of rats as previously described(30). In short, following sacrificing the rats, the inflamed hind paw tissues of rats were quickly removed and fixed in buffered 10 % formalin for a day. Dipping the paw tissues into alcohol to remove the water, and then the paw tissues of rats were embedded in paraffin. The sections of paw tissues were cut and stained using HE staining, and visualized with a light microscope. Subsequently, the degree of inflammation of rats left hind paw tissues was quantified with white blood cell count.

CatWalk-automated gait analysis

The CatWalk system (XT, Noldus Information Technology, Netherlands) was used for evaluating motor function defects and the quantitative examination of gait parameters alteration induced by chronic pain

in rodents, when they walk naturally (without compulsion). The CatWalk system provides an objective and accurate assessment of limb coordination, and it has been used in inflammatory pain study(31). This system mainly includes a glass plate with a built-in fluorescent lamp, a high-frequency camera, image conversion, transmission system, and hardware facilities. The experiment was conducted in the darkroom environment, try to avoid the influence of the computer display screen on the instrument data collection and reduce food temptation and other interference factors. When rats walked from one end of the walking platform to the other end, the footprints were captured by the high-speed camera under the walking platform and analyzed by software. Before the formal experiment, rats need to be received pre-training (three measurement with once daily for 3 consecutive days) so that they can run the whole course within 10 s. If rats still cannot pass the training, they will be removed from the experiment, and the pre-training and detection will be carried out at a fixed time point. The time points of measurement were day 1 before and day 21 following CFA injection. In this study, the CatWalk gait parameters were selected as follows: stand time (s) is the duration of ground contact for a single paw; swing time (s) is the duration of no contact of a paw with the glass plate in a step cycle; swing speed (m/s) is the ratio of stride length to swing duration during swing phase, and print area (cm²) represents the surface area of the complete print of a paw.

Molecular docking

The crystal structure of the $\alpha 7nAChR$ protein was obtained by Brookhaven protein database, and the water molecules and original ligands were removed by Chimera program. Then Adt software was used to add charge and non-polar hydrogen to $\alpha 7nAChR$, and set the Mesh of receptor molecule $\alpha 7nAChR$ with original ligand lobeline and ICTF respectively. Using inhibitory constant (K_i) and binding energy (ΔG) as reference parameters and lobeline as positive control, the K_i and ΔG values of ICTF active components docking with $\alpha 7nAChR$ were obtained, and the conformations were analyzed.

Intrathecal injection

Following rats were anesthetized using 2% -3% isoflurane, the implantation of intrathecal cannula to rats were conducted as previously described(32). Briefly, a PE-10 polyethylene catheter (BB31695-PE/1, American Health & Medical Supply International Corp, USA) was implanted between the L5 and L6 vertebrae to the lumbar enlargement level (3.5-4 cm rostral than the cannula) of the spinal cord. The outer part of the PE-10 catheter was plugged and fixed onto the skin between rat ears to close the wound. All surgery protocols were performed under sterile state. Two days post-surgery, only rats without the sign of spinal cord injury were applied in this experiment. Before further experimental procedure was performed, the rats were given a 5-day recovery, and then the rat model of CFA-induced inflammatory pain was established. In the research, after ICTF was treated to CFA rats on day 14, $\alpha 7nAChR$ antagonist α -Btgx was intrathecally injected for 7 consecutive days with once daily at a dose of 1.0 $\mu\text{g}/\text{kg}$ (33). Intrathecal injection of α -Btgx or PBS through the implanted catheter with a 20 μl volume of solution, and then using 10 μl PBS to flush. The needle kept in situ for 2 min after each injection.

Quantitative RT-PCR analysis

After deep anesthesia with 2–3% isoflurane, the fresh lumbar (L4-L6) dorsal spinal cord of rat were rapidly collected in 1.5ml centrifuge tube and stored in liquid nitrogen. Total RNA was isolated from the spinal cord segments at L4-L6 of rat and prepared for quantitative real-time polymerase chain reaction (qRT-PCR) as described in our previous report(34). The primer sequences for qRT-PCR were provided by Invitrogen (USA), the primer sequences used in this experiment were as the follows: α 7nAChR, sense CCCTGGCTCTGCTGGTATTC, antisense TGGTGCTGGCGAAGTATTGT; HMGB1, sense GAACAACACTGCTGCGGATG, antisense CCCTTTTTTCGCTGCATCAGG; NF- κ B, sense GAAGACACGAGGCTGCAACT, antisense TTTTAGAAGGGGCGGGACTC; IL-1 β , sense GGGATGATGACGACCTGCTA, antisense CCACTTGTTGGCTTATGTTCTG; IL-10, sense CTTACTGACTGGCATGAGGATCA, antisense GCAGCTCTAGGAGCATGTGG; GAPDH, sense CAGCCGCATCTTCTTGTGC, antisense GGTAACCAGGCGTCCGATA. Quantitative RT-PCR data were normalized with GAPDH mRNA level which was regarded as a control, and the relative mRNA level was expressed as $2^{-\Delta\Delta Ct}$ values.

Western blot analysis

In this study, western blot assay was performed as described in previous reports(35). After rats were anesthetized by using 2–3% isoflurane, and the spinal cord was dissected and isolated immediately and rinsed in ice-cold PBS. Proteins were extracted from the spinal cord segments at L4-L6. Thirty micrograms of protein per sample were denatured and then separated with 10% SDS-PAGE and western-blotted on a PVDF (Millipore, CA) membrane using a minigel and mini transblot apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 60 min at room temperature, and subsequently the membrane respectively immunolabelled overnight at 4°C with antibodies of rabbit anti- α 7nAChR (1:1000, ab216485 Abcam, UK), rabbit anti-HMGB1 (1:2000, ab79823, Abcam, UK), rabbit anti-NF- κ B p65 (1:1000, 4764s, CST, UK) and rabbit anti-phospho-NF- κ B p65 (1:1000, 3033s, CST, UK), anti-IL-1 β (1:1000, ab9789, Abcam, UK), anti-IL-10 (1:1000, ab9969, Abcam, UK), mouse anti-GAPDH (1:1000, M1000140, Solarbio, China). The blots were washed with Tris-buffered saline and Tween, and then incubated with the horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:1000, Cell Signaling Technology, USA) for 60 min at 4°C. The blots site of the antigen-antibody complex was visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA). The bands were analyzed using Quantity One software (Bio-Rad). GAPDH was used as the internal control. The standardized ratio of α 7nAChR, HMGB1, phospho-NF- κ B, IL-1 β , and IL-10 protein to GAPDH band density was utilized to calculate the change of corresponding protein expression level.

Immunofluorescence staining

After assessment of pain-related behavioral alterations, rats were anesthetized by using 2–3% isoflurane, and the thoracic cavity was incised to expose the heart. A puncture needle was inserted from the left apex and extended towards the aorta. The right auricle was incised and the rat was transcordially perfused with 0.9% normal saline and then 4% paraformaldehyde solution in 0.1 M phosphate-buffered saline (PBS) (pH 7.4, 4°C). Immunofluorescence staining was performed as described in the previous

studies(36). Briefly, the spinal cord segments at L4-L6 were immediately removed and put into 4% paraformaldehyde solution for 4 h, followed by cryoprotection in 20% and 30% sucrose, respectively, at 4°C until it submerged. Transverse sections (10 µm) were cut on a cryostat, and blocked with 5% BSA for 60 min at room temperature, after washing with PBS for 15 min and incubated with 5% BSA for overnight at 4°C, the sections were incubated in the antibody solution containing rabbit anti-α7nAChR (1:300, ab216485, Abcam, UK) and mouse anti-Iba1 for microglia (1:300, 3256633, Merck millipore, USA), respectively. After washing with PBS for 15 min, the sections were incubated for 2 h at room temperature with Alexa Fluor™ 555 goat anti-rabbit IgG (H + L) (1:1000, A21429, invitrogen, USA), and Alexa Fluor™ 488 goat anti-mouse IgG (H + L) (1:1000, A11029, invitrogen, USA). All stained sections were detected and analyzed with a confocal laser scanning fluorescence microscope.

Statistical analysis

Statistical analyses were conducted with Prism 8.0 software. All experimental data are expressed as the mean ± SEM. The data differences between groups were compared using one-way ANOVA followed by Newman–Keuls post-hoc test or two-way ANOVA followed by Bonferroni post-hoc test. $P < 0.05$ was considered as statistically significant.

Results

ICTF inhibits CFA-induced pain hypersensitivity and targets α7nAChR in rats

In order to determine the effective dose of ICTF on CFA-induced inflammatory pain, ICTF at the doses of 0.3, 1.0, and 3.0 mg/kg were selected for this research. The paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) in rats were detected on day 1 before and day 1, 3, 7, 10, and 14 following CFA injection, respectively. The results are shown in Fig. 2B-C. Compared with the Ctrl group, the PWT and PWL of rats were significantly reduced in the CFA group on day 1 after CFA injection ($P < 0.05$, $P < 0.01$, $P < 0.001$), suggesting that a rat model of CFA-induced inflammatory pain was successfully established. We further observed that ICTF at the doses of 0.3 and 1.0 mg/kg had no significant effect on the PWT and PWL in CFA rats. While 3.0 mg/kg of ICTF obviously enhanced the PWL and PWT on day 14 after CFA injection ($P < 0.05$). These results indicated that ICTF (3.0 mg/kg) could alleviate CFA-induced pain hypersensitivity, thereby 3.0 mg/kg of ICTF was chosen for further study. Subsequently, The binding pattern and affinity between ICTF and α7nAChR were predicted by molecular docking. The chemical structure of ICTF is similar to α7nAChR agonist lobeline, the data of docking is shown in Fig. 2D. We found that the inhibitory constants (K_i) and the binding energy (ΔG) of ICTF and α7nAChR were similar to lobeline. Then we analyze the combination of lobeline and ICTF with α7nAChR. From the best conformations of docking, the docking of lobeline and α7nAChR is shown in Fig. 2E. ICTF can stably bind to the active region of α7nAChR (Fig. 2F), the active sites of ICTF and α7nAChR are matched from space. Based on this, indicating that α7nAChR may be a potential target for lobeline and ICTF (Fig. 2G).

Effects of ICTF on CatWalk gait parameters in CFA rats

The experiment evaluated gait parameters in CFA-induced inflammatory pain with CatWalk gait analysis that regarded as an objective method. In this examination, we detected several gait parameters such as stand time (s), swing time (s), swing speed (cm/s), and print area (cm²) to exhibit pain-related behaviors in CFA rats. As shown in Fig. 3A-F, the CatWalk gait analysis was performed after treatment of ICTF (3.0 mg/kg, i.p.) to CFA rats on day 1 before and day 21 after CFA injection, respectively. Compared with the Ctrl group, Injection of CFA significantly reduced the gait parameters including stand time (s), swing speed (cm/s), and print area (cm²) but increased swing time (s) on day 21 post-CFA injection ($P < 0.05$), suggesting that CFA provoked painful sensory and motor function disorder in rats. However, ICTF treatment (3.0 mg/kg) obviously reversed the alterations of gait parameters induced by CFA injection in rats ($P < 0.05$) (Fig. 3A-C, E). Additionally, the results of 2D images also exhibited that compared with the CFA group, LH stand time was longer but LH swing time was shorter in the CFA + ICTF group (Fig. 3D). Further, the effective images of paw print area showed that LH paw print of rats in the Ctrl group was more clear and completed, while only a small part of LH paw print was got in the CFA group, and LH paw print area was markedly reduced. After ICTF treatment for 21 days, LH paw print area of rats in the CFA + ICTF group was obviously enhanced compared to the CFA group (Fig. 3F). These results demonstrated that ICTF is effective to alleviate pain-related behaviors as indicated by the changes of CatWalk gait parameters in CFA rats.

Effects of ICTF on the paw edema volume and paw tissue inflammation in CFA rats

This research investigated the effects of ICTF on CFA-induced paw edema volume and paw tissue inflammation in rats. Firstly, following treatment of ICTF (3.0 mg/kg, i.p.), the paw edema volume of rats was examined on day 1 before and day 1, 7, 14, and 21 after injection of CFA, respectively. As shown in Fig. 4A, compared with the Ctrl group, the paw edema volume in the CFA group was markedly enhanced starting from day 1 and still retained to day 21 after CFA injection ($P < 0.001$). While ICTF treatment significantly decreased the paw edema volume of CFA rats from day 14 and retained to day 21 post-CFA injection ($P < 0.05$, $P < 0.01$). Secondly, compared with the Ctrl group, CFA effectively elicited swelling on the plantar surface of the injected paw on day 1 after CFA injection, and paw swelling became more severe with obvious inflammatory response on day 7, 14, and 21 post-CFA injection. However, the paw swelling gradually reduced from day 14 following ICTF treatment of CFA rats (Fig. 4B). Thirdly, the assessment of HE staining demonstrated that rats in the Ctrl group that suffered injection of normal saline to the left hind paw had normal paw tissue (Fig. 4C). Conversely, compared with the Ctrl group, the left hind paw of CFA rats exhibited massive accumulation of inflammatory cells (Fig. 4C). While the inflammatory cells were markedly decreased by treatment of CFA rats with ICTF (3.0 mg/kg) on day 21 post-CFA injection (Fig. 4C). Additionally, the degree of inflammatory response was determined by inflammation scoring, this suggested that administration of ICTF (3.0 mg/kg) enabled to reduced CFA-induced inflammation (Fig. 4D).

Effect of ICTF on co-expression of spinal $\alpha 7$ nAChR and IBA-1 in CFA rats

To determine the possible role of ICTF on the relationship between $\alpha 7$ nAChR and microglial activation in the spinal cord of CFA rats, the co-expression of $\alpha 7$ nAChR and IBA-1 which is a molecular indicator of microglial activation in the spinal dorsal horn of ICTF-treated rat on day 21 post-CFA injection was detected by immunofluorescence staining. As shown in Fig. 5, compared with the Ctrl group, it was found that spinal $\alpha 7$ nAChR expression was obviously decreased in CFA group, however, prominent up-regulation of spinal $\alpha 7$ nAChR expression after ICTF (3.0 mg/kg) treatment were observed (Fig. 5B). Similarly, in comparison with the Ctrl group, CFA induced the elevation of IBA-1 expression induced by CFA, but this effect was inhibited by ICTF treatment of CFA rats (Fig. 5C). In addition, we further found that CFA evoked the co-expression of $\alpha 7$ nAChR with IBA-1 in the spinal cord with double immunofluorescence staining (Fig. 5A). In contrast, the co-expression of spinal $\alpha 7$ nAChR with IBA-1 was reversed by ICTF treatment of CFA rats.

α -Bgtx reversed the inhibitory effects of ICTF on CFA-induced pain hypersensitivity in rats

In this experiment, we further explored the time-course effect of ICTF (3.0 mg/kg) on CFA-induced inflammatory pain in rats. The results are shown in Fig. 6A. Compared with the Ctrl group, the PWT in the CFA group was significantly reduced ($P < 0.001$). After ICTF treatment, the PWT of rats in the CFA + ICTF group was markedly increased compared to the CFA group from day 14 and retained to day 21 post-CFA injection ($P < 0.05$, $P < 0.01$). Similarly, Compared with the Ctrl group, the PWL in the CFA group was obviously decreased ($P < 0.001$). While compared with the CFA group, the PWL of rats in the CFA + ICTF group was markedly enhanced from day 14 and retained to day 21 post-CFA injection ($P < 0.05$, $P < 0.01$) (Fig. 6B). These data demonstrated that ICTF effectively attenuates pain hypersensitivity in CFA-induced inflammatory pain rats. In order to further define whether the involvement of $\alpha 7$ nAChR in the anti-nociceptive effect of ICTF on CFA-induced pain hypersensitivity in rats, intrathecally injected 1.0 μ g/kg $\alpha 7$ nAChR antagonist α -Bgtx or PBS on CFA rats starting from day 14 of ICTF treatment for 7 consecutive days with once daily. The results are shown in Fig. 6C-D. Compared with intrathecal administration of PBS, the PWT and PWL of rats given α -Bgtx were markedly reduced ($P < 0.001$). The findings revealed that α -Bgtx could reverse the inhibitory effects of ICTF on CFA-induced inflammatory pain in rats.

α -Bgtx reversed ICTF-induced the up-regulation of $\alpha 7$ nAChR protein expression in the spinal cord of CFA rats

In order to evaluate the possible effect of ICTF on spinal $\alpha 7$ nAChR in CFA rats, qRT-PCR and western blot assay were applied to detect the mRNA and protein levels of $\alpha 7$ nAChR in the spinal cord of CFA rats on day 21 after ICTF treatment. As shown in Fig. 7A, compared with the Ctrl group, the mRNA expression of $\alpha 7$ nAChR in the spinal cord of CFA rats was obviously decreased on day 21 after CFA injection ($P <$

0.001). Following ICTF was given to CFA rats, the level of spinal $\alpha 7nAChR$ mRNA in the CFA + ICTF group was markedly up-regulated ($P < 0.001$). Similarly, as shown in Fig. 7B, compared with the Ctrl group, the protein expression of $\alpha 7nAChR$ in the spinal cord of the CFA group rats was significantly down-regulated on day 21 after CFA injection ($P < 0.01$). Following ICTF treatment, the protein level of spinal $\alpha 7nAChR$ in the CFA + ICTF group was markedly increased ($P < 0.001$). Indicating that ICTF could enhance the expression levels of $\alpha 7nAChR$ mRNA and protein in the spinal cord of CFA rats. To further define whether the effect of ICTF on CFA-induced inflammatory pain is through $\alpha 7nAChR$ signal in rats, on day 14 of ICTF administration to CFA rats, intrathecal injection of 1.0 $\mu\text{g}/\text{kg}$ $\alpha\text{-Bgtx}$ or PBS for 7 consecutive days, and then collected the spinal cord of CFA rats on day 21 to examine $\alpha 7nAChR$ protein expression. As shown in Fig. 7C. Compared with intrathecal injection of PBS, the protein level of $\alpha 7nAChR$ in the spinal cord of CFA rats following administration of $\alpha\text{-Bgtx}$ was obviously down-regulated ($P < 0.001$). These data demonstrated that $\alpha\text{-Bgtx}$ could reverse the enhancement of $\alpha 7nAChR$ expression induced by ICTF in CFA rats.

$\alpha\text{-Bgtx}$ reversed ICTF-induced down-regulation of HMGB1 mRNA and protein levels in the spinal cord of CFA rats

In order to determine the potential effect of ICTF on spinal HMGB1 in CFA rats, on day 21 after CFA injection, qRT-PCR and western blot assay were used to examine the mRNA and protein expression of spinal HMGB1 by ICTF treatment in CFA rats. As shown in Fig. 8A. Compared with the Ctrl group, the mRNA expression of spinal HMGB1 in the CFA group was significantly up-regulated ($P < 0.001$). Compared with the CFA group, the mRNA level of spinal HMGB1 in the CFA + ICTF group was markedly decreased ($P < 0.001$). Similarly, as shown in Fig. 8B, compared with the Ctrl group, the protein expression of spinal HMGB1 in the CFA group rats was obviously increased ($P < 0.001$), while compared with the CFA group, the protein expression of HMGB1 in the spinal cord of CFA + ICTF group rats was markedly down-regulated ($P < 0.001$). These data revealed that ICTF effectively suppressed the HMGB1 mRNA and protein expression in the spinal cord of CFA rats. To further evaluate the effect of $\alpha\text{-Bgtx}$ on HMGB1 in the spinal cord of CFA rats by ICTF treatment, following ICTF administration, we intrathecally given $\alpha\text{-Bgtx}$ or PBS to CFA rats from day 14, and the spinal cord of CFA rats was conducted to determine HMGB1 protein expression on day 21. As shown in Fig. 8C, compared with intrathecal injection of PBS, the level of HMGB1 protein in the spinal cord of CFA rats was significantly up-regulated by intrathecally injected 1.0 $\mu\text{g}/\text{kg}$ $\alpha\text{-Bgtx}$ ($P < 0.001$). These results indicated that $\alpha\text{-Bgtx}$ successfully reversed the inhibitory effect of ICTF on spinal HMGB1 in CFA rats.

$\alpha\text{-Bgtx}$ reversed ICTF-induced inhibition of NF- κB signaling activation in the spinal cord of CFA rats

To evaluate the possible effect of ICTF on NF- κB signaling activation in the spinal cord of CFA rats, on day 21 following CFA injection, qRT-PCR and western blot assay were used to detect the mRNA and protein expression of spinal NF- κB by ICTF treatment in CFA rats. These results were shown in Fig. 8D.

Compared with the Ctrl group, mRNA level of spinal NF- κ B p65 in the CFA group was markedly increased ($P < 0.05$). Compared with the CFA group, mRNA level of spinal NF- κ B p65 in the CFA + ICTF group was obviously down-regulated ($P < 0.001$). Similarly, as shown in Fig. 8E, compared with the Ctrl group, the protein level of spinal p-p65 in the CFA group rats was significantly enhanced ($P < 0.05$), while compared with the CFA group, the protein expression of spinal p-p65 in the CFA + ICTF group was obviously down-regulated ($P < 0.05$). These results showed that ICTF could reduce NF- κ B p65 mRNA and p-p65 protein levels in the spinal cord of CFA rats. To further determine the effect of α -Bgtx on NF- κ B signaling in the spinal cord of CFA rats by ICTF treatment, after ICTF treatment of CFA rats for 14 days, we intrathecally injected α -Bgtx or PBS to CFA rats from day 14, and the spinal cord of CFA rats was obtained to examine the protein level of p-p65 on day 21. As shown in Fig. 8F, compared with PBS group, after intrathecal injection of 1.0 μ g/kg α -Bgtx, p-p65 protein expression in the spinal cord of CFA rats was markedly increased ($P < 0.01$). These findings demonstrated that α -Bgtx effectively reversed the inhibitory effect of ICTF on NF- κ B signaling activation in the spinal cord of CFA rats.

α -Bgtx reversed ICTF-induced reduction of IL-1 β mRNA and protein expression in the spinal cord of CFA rats

To elucidate the potential role of ICTF on spinal IL-1 β in CFA rats, on day 21 after CFA injection, qRT-PCR and western blot assay were applied to detect the mRNA and protein levels of spinal IL-1 β by ICTF treatment in CFA rats. As shown in Fig. 9A. Compared with the Ctrl group, the mRNA expression of spinal IL-1 β in the CFA group was significantly enhanced ($P < 0.05$). Compared with the CFA group, the mRNA level of spinal IL-1 β in the CFA + ICTF group was obviously down-regulated ($P < 0.05$). Consistently, as shown in Fig. 9B, compared with the Ctrl group, the protein level of spinal IL-1 β in the CFA group rats was obviously up-regulated ($P < 0.001$), while compared with the CFA group, the protein expression of spinal IL-1 β in CFA + ICTF group rats was significantly decreased ($P < 0.001$). The findings demonstrated that ICTF effectively reduced IL-1 β mRNA and protein expression in the spinal cord of CFA rats. In order to further assess the possible effect of α -Bgtx on spinal IL-1 β in CFA rats by ICTF, after ICTF treatment, intrathecal injection of α -Bgtx or PBS to CFA rats from day 14, and the spinal cord was collected to detect the protein level of IL-1 β on day 21. As shown in Fig. 9C, we observed that compared with PBS group, intrathecally given 1.0 μ g/kg α -Bgtx markedly enhanced IL-1 β protein expression in the spinal cord of CFA rats ($P < 0.001$). These data revealed that α -Bgtx successfully reversed the inhibitory effect of ICTF on spinal IL-1 β in CFA rats.

α -Bgtx reversed ICTF-induced enhancement of IL-10 mRNA and protein levels in the spinal cord of CFA rats

In order to define the possible effect of ICTF on spinal IL-10 in CFA rats, on 21 day after injected CFA, qRT-PCR and western blot assay were used to determine the mRNA and protein levels of spinal IL-10 by ICTF in CFA rats. These data were shown in Fig. 9D. Compared with the Ctrl group, mRNA level of spinal IL-10

in the CFA group was obviously decreased ($P < 0.01$). Compared with the CFA group, the mRNA expression of spinal IL-10 in the CFA + ICTF group was markedly enhanced ($P < 0.01$). Similarly, as shown in Fig. 9E, compared with the Ctrl group, the protein expression of spinal IL-10 in the CFA group rats was significantly down-regulated ($P < 0.01$), while compared with the CFA group, the protein level of spinal IL-10 in the CFA + ICTF group was obviously up-regulated ($P < 0.01$). These results revealed that ICTF effectively increased IL-10 mRNA and protein levels in the spinal cord of CFA rats. To further evaluate the potential role of α -Bgtx on spinal IL-10 in CFA rats by ICTF treatment, after ICTF administration, intrathecally injected α -Bgtx or PBS to CFA rats from day 14, and the spinal cord was obtained to examine the protein expression of IL-10 on day 21. As shown in Fig. 9F, we observed that compared with PBS group, intrathecally injected 1.0 μ g/kg α -Bgtx significantly decreased IL-10 protein level in the spinal cord of CFA rats ($P < 0.001$). These results indicated that α -Bgtx effectively reversed the inhibitory effect of ICTF on spinal IL-10 in CFA rats.

Discussion

Since there is little evidence that the effect of ICTF on chronic inflammatory pain and its underlying mechanisms. For this purpose, our experiment was designed to investigate the potential inhibitory effects of ICTF on CFA-induced inflammatory pain in rats, we then highlighted whether involvement of spinal α 7nAChR, HMGB1/NF- κ B signaling, and inflammatory cytokines in ICTF alleviating pain-related behaviors in CFA rats. In this study, we found that ICTF exerted anti-nociceptive and anti-inflammatory effects on CFA-induced pain hypersensitivity through inhibiting HMGB1/NF- κ B signaling pathway following activation of α 7nAChR-dependent in the spinal cord of rats. Further molecular docking assay demonstrated that ICTF targeted α 7nAChR for suppressing CFA-induced inflammatory pain. Findings from this experiment might provide new insights into the mechanisms underlying ICTF being acted as a potential therapeutic candidate for CFA-induced inflammatory pain.

It is generally accepted that chronic inflammatory pain is an increasingly severe global public health problem and greatly impacts patient's life quality(1). Currently available and conventional analgesics with unsatisfactory efficacy for inflammatory pain relief including non-steroidal anti-inflammatory drugs (NSAID) and opioids(20, 37). It is thus critical to uncover and develop novel high-efficacy and low-toxicity interventions against chronic inflammatory pain. More importantly, traditional Chinese medicine is proved to be effective candidates for chronic pain relief(38). We previously confirmed that ICTF exerts a neuroprotective role by suppressing excitotoxicity and cerebral ischemia-reperfusion injury(24). It is known that neuroinflammation is a crucial and inevitable pathogenesis process in mediating inflammatory pain development(6). Thereby, in this study, provoking us to apply a rat model of CFA-induced inflammatory pain to define whether ICTF has an anti-nociceptive effect in CFA rats. Our results showed that both PWT and PWL were significantly reduced on day 1 after injected CFA, indicating that CFA-induced inflammatory pain rat model was successfully established. While ICTF could dose-dependently inhibit CFA-induced mechanical allodynia and thermal hyperalgesia. Further, we explored the time-course effect of ICTF (3.0 mg/kg) in CFA-induced inflammatory pain rats. It is found that ICTF treatment of CFA rats effectively enhanced both PWT and PWL from day 14 and retained until day 21

post-CFA injection. These findings suggested that ICTF exhibited an anti-nociceptive effect on CFA-induced inflammatory pain.

Nowadays, CatWalk gait analysis that obtained an objective and quantitative assessment of gait parameters has been utilized to determine the pain hypersensitivity in neuropathic pain(39). Thus, we hypothesized that CatWalk gait analysis may provide an interesting complementary option to evaluate the alterations of motor function related to CFA-induced pain hypersensitivity. This study was designed to apply CatWalk gait analysis to elucidate the possible effects of ICTF on gait parameters in CFA rats. We currently observed that CFA obviously reduced the gait parameters involving stand time, swing speed, and print area but increased swing time on day 21 post-CFA injection, suggesting that CFA elicited painful sensory and motor function disorder in rats. While ICTF could reverse CFA-induced alterations of gait parameters. In addition, 2D images further exhibited that after ICTF treatment of CFA rats, the stand time was longer but swing time was shorter, as well as paw print area was markedly enhanced. The results from CatWalk gait analysis strongly supported that ICTF indeed had an inhibitory effect of pain-related behaviors induced by CFA in rats.

It is reported that sensitization of peripheral nociceptors by pro-inflammatory mediators is a major pathogenesis of inflammatory pain(40). During inflammation, pro-inflammatory mediators are always released into the damage tissues, resulting in redness and swelling of inflamed areas(41). Injection of CFA into rodent paw plantar is often utilized to establish a chronic inflammatory pain model owing to eliciting inflammatory responses, such as swelling, inflammatory infiltrate formation, and pro-inflammatory mediators release(42), implying that reduction of pro-inflammatory mediators is an effective therapeutic approach for alleviating inflammatory pain. Thereby, our research explored whether ICTF has anti-inflammatory effect on CFA-induced paw edema volume and paw tissue inflammation in rats. CFA-induced paw edema volume markedly enhanced starting from day 1 and retained until day 21 post-CFA injection, while ICTF could reduce the paw edema volume of CFA rats beginning from day 14 post-CFA injection. Further, the photographs exhibited that CFA provoked obvious swelling on the plantar surface of the injected paw starting from day 1 and paw swelling became more severe from day 7, 14, and 21 post-CFA injection, whereas the paw swelling of CFA rats gradually reduced from day 14 following ICTF treatment. Additionally, HE staining showed that CFA produced massive accumulation of inflammatory cells in CFA rats, while the inflammatory cells were markedly reduced by ICTF treatment on day 21 post-CFA injection. This is consistent with other results(43). Altogether, the data supported the notion that ICTF has profound anti-inflammatory and anti-edema effects on CFA-induced inflammation.

Based on the above findings, we postulated that ICTF may be served as a promising therapeutic reagent against CFA-induced inflammatory pain, but the precise mechanism underlying the inhibitory effects of ICTF on CFA rats need further investigation. The nervous system interacting with immune system is crucial for modulating neuroimmune responses and controlling inflammation(44, 45). Within this process, cholinergic anti-inflammatory pathway regulates the inflammatory response through the activation of $\alpha 7$ nAChR-dependent(46). Further, $\alpha 7$ nAChR was identified to mainly express on pain transmission pathways such as spinal cord, which mediated anti-inflammatory effects by down-regulating pro-

inflammatory mediators(47, 48). Intraplantar injection of CFA induces neuroinflammation and inflammatory pain, and exhibits more edema, hyperalgesia and allodynia in the $\alpha 7$ nAChR KO mice compared with the wild-type(49). Previously studies have shown that activating spinal $\alpha 7$ nAChR exerts anti-inflammatory and analgesic effects through modulation of pro-inflammatory cytokines, and thus alleviates pain hypersensitivity in CFA rats(50). Further, Our recent data have confirmed that 2 Hz EA stimulation can attenuate mechanical hypersensitivity in SNI rats by up-regulating the mRNA and protein expression of spinal $\alpha 7$ nAChR, and reducing the pro-inflammatory cytokine IL-1 β (33). These results supported that $\alpha 7$ nAChR maybe a new target for treating chronic pain in the spinal cord. Thereby, this indeed provides novel option for the utility of activating $\alpha 7$ nAChR signal in the chronic pain relief. In our study, the expression of spinal $\alpha 7$ nAChR mRNA and protein dramatically decreased on day 21 after given CFA, and immunofluorescence staining also showed that expression of $\alpha 7$ nAChR reduced in the spinal dorsal horn of CFA rats, suggesting that spinal $\alpha 7$ nAChR participated in the modulation of CFA-induced inflammatory pain. This is consistent with other reports. While following ICTF treatment, the levels of spinal $\alpha 7$ nAChR mRNA and protein significantly enhanced on day 21 after CFA injection. These results are consistent with the alleviation of CFA-induced mechanical allodynia and thermal hyperalgesia by ICTF treatment. Further, immunofluorescence results exhibited that TCTF treatment also increased the expression of $\alpha 7$ nAChR in the spinal dorsal horn of CFA rats, implying that $\alpha 7$ nAChR might mediate the anti-nociception of ICTF in the spinal cord of CFA rats. To further elucidate the effect of spinal $\alpha 7$ nAChR on ICTF-mediated anti-nociception and its possible mechanism in CFA rats, intrathecally injected $\alpha 7$ nAChR antagonist α -Bgtx was utilized. We found that α -Bgtx successfully reversed the inhibitory effects of ICTF on CFA-induced pain hypersensitivity and the reduction of $\alpha 7$ nAChR protein level induced by CFA in the spinal cord of rats, suggesting an suppression of ICTF-provoked anti-nociception to CFA-induced inflammatory pain. Collectively, our research indicates that the spinal $\alpha 7$ nAChR is implicated in the attenuating CFA-induced pain hypersensitivity mediated by ICTF treatment in CFA rats.

It is clear that the mechanism underlying inflammatory pain is highly complex, such as neuroinflammation induced by pro-inflammatory mediators(51). As a pro-inflammatory mediator, HMGB1 is highly expressed in the spinal cord and exhibits an important role in the inflammation response(52), suggesting that spinal HMGB1 signal contributes to neuroinflammation and inflammatory pain development. Therefore, whether the anti-inflammatory effect of ICTF through inhibiting HMGB1 signal is an interesting question that remains to be fully defined. In our study, the mRNA and protein expression of spinal HMGB1 was markedly increased in CFA rats, but ICTF treatment of CFA rats obviously reduced spinal HMGB1 mRNA and protein levels, indicating that ICTF exerts anti-inflammatory effect may through inhibiting spinal HMGB1 signal in CFA rats. It is known that $\alpha 7$ nAChR-dependent cholinergic signaling is implicated in suppressing the release of HMGB1(16). For example, under inflammatory pain states, the application of agonist triggering $\alpha 7$ nAChR signal effectively block the release of HMGB1(53), conversely, administration of $\alpha 7$ nAChR inhibitor α -Bgtx can completely reverse this effect(54). Suggesting that $\alpha 7$ nAChR-HMGB1 signaling may be involved in modulating chronic inflammatory pain. Further, we determine the effect of α -Bgtx on spinal HMGB1 level by ICTF treatment in CFA rats, after intrathecally injected α -Bgtx to CFA rats, spinal HMGB1 protein expression in CFA rats was significantly enhanced,

indicating that α -Bgtx successfully reversed the inhibitory effect of ICTF on spinal HMGB1 in CFA rats. The data supported that ICTF exhibits anti-nociception on CFA-induced inflammatory pain by inhibiting HMGB1 cascade via the activation of α 7nAChR.

Evidence showed that blockade of HMGB1/NF- κ B signaling could alleviate CFA-induced inflammatory pain(17), implying a promising therapeutic approach for inflammatory pain by targeting HMGB1/NF- κ B signaling. Notably, the inflammatory response has been identified as a vital mechanism underlying the pathogenesis of inflammatory pain(55). It is reported that NF- κ B, a critical nuclear transcription factor, plays an important role in the modulation of the inflammatory response(56). The activated NF- κ B p65 is translocated to the nucleus and promoted the transcription of target genes including pro-inflammatory cytokines(57). It is known that activation of NF- κ B signal mediated neuroinflammation by increasing pro-inflammatory cytokines expression. For example, within the inflammatory pain, NF- κ B p65 expression was found to be enhanced(30), conversely, inhibition of NF- κ B cascade can attenuate inflammatory pain. Suggesting that NF- κ B cascade plays an important role in inflammatory pain. Consistent with other reports(17), our present results showed that CFA markedly enhanced mRNA and protein levels of spinal NF- κ B p65, while it was restored by ICTF treatment. It is clear that activation of α 7nAChR can reduce NF- κ B activity and suppress inflammatory response(16). In this study, α -Bgtx effectively reverse the inhibitory effect of ICTF treatment on spinal NF- κ B signal in CFA rats, indicating that α 7nAChR-mediated suppression of spinal NF- κ B activation is implicated in the inhibitory effects of ICTF on CFA-induced inflammatory pain.

It is well known a role of neuroinflammation that closely associated with pro-inflammatory mediators and microglial activation in the pathogenesis of inflammatory pain(7). Spinal microglia is indicated to be a critical resident immune cell and plays an essential role in the inflammatory response(58). The activated microglia triggers pro-inflammatory cytokines release, such as IL-1 β , IL-6, and TNF- α (59), conversely, the pro-inflammatory cytokines can also activate microglia(60). Microglia has been acted as a key initiator in inflammatory pain development(61). For instance, the activation of microglia within the spinal cord following inflammatory pain was well recognized to produce pain hypersensitivity(62). Microglial activation leads to an enhancement of pro-inflammatory cytokines to maintain central sensitization and inflammatory pain(63), thereby the blockade of microglial activation maybe a novel management for inflammatory pain. In the spinal cord, we presently applied the microglial marker IBA-1 to assess the activation of microglia in CFA-induced inflammatory pain. A obvious up-regulation of spinal IBA-1 expression was found in CFA rats, indicating that CFA induced the activation of microglia in the spinal cord. Additionally, CFA also produced an enhancement of spinal IL-1 β in mRNA and protein expression. These data further supported the notion that microglial activation is the main source of pro-inflammatory cytokines in inflammatory pain(59). While ICTF treatment markedly reduced the enhancement of spinal IBA-1 expression induced by CFA, and decreased the level of spinal IL-1 β in CFA rats. Thus, we postulate that the anti-inflammatory effects of ICTF on CFA-induced inflammatory pain may through suppressing both microglial activation and IL-1 β in the spinal cord.

As previously reported, the interactions between the nervous and immune systems participate in modulating chronic pain(64). The release of pro-inflammatory mediators and the loss of anti-inflammatory cytokines result in neuroimmune response(65), indicating that an imbalance in the cytokines micro-environment mediates regulating inflammatory pain. It is clear that some well-known pro-inflammatory cytokines including IL-1 β , IL-6, and TNF- α are implicated in inflammatory pain(51), conversely, IL-10 is identified as a profound anti-inflammatory cytokine that is greatly expressed on microglia, which exhibits marked anti-nociception in inflammatory pain(66). For example, IL-10 can suppress CFA-induced inflammatory pain by reduction of pro-inflammatory cytokines(66, 67), and treatment of IL-10 to inflammatory pain rats effectively provoked an anti-nociceptive effect in the spinal cord(17). Presently we also noticed that CFA obviously produced an elevation in IL-1 β mRNA and protein levels, and together with a significant reduction of IL-10 mRNA and protein expression in the spinal cord, implying that an imbalance between pro-inflammatory cytokines (IL-1 β) and anti-inflammatory cytokine (IL-10) in the spinal cord triggered CFA-induced inflammatory pain. We currently found that ICTF treatment markedly reversed the enhancement of IL-1 β protein level and the increase of IL-10 protein expression induced by CFA in the spinal cord. Thus, ICTF has anti-inflammatory activity maybe via re-balancing the cytokine micro-environment in the spinal cord of CFA rats. Increasing evidence have reported that α 7nAChR inhibits inflammation response by decreasing pro-inflammatory cytokines(68). In the spinal cord, α 7nAChR is abundantly expressed on microglia, which is proven to induce and maintain chronic inflammatory pain. More strikingly, we presently discovered that intrathecal injection of α -Bgtx could antagonize the decrease of IL-1 β protein level and the elevation of IL-10 protein expression induced by ICTF treatment in the spinal cord of CFA rats. Our current findings further highlighted that ICTF relieves inflammatory pain by reducing IL-1 β expression and increasing IL-10 level via activating α 7nAChR in the spinal cord of CFA rats.

Conclusion

In summary, in this study, we have provided findings that ICTF effectively afforded profound anti-nociceptive and anti-inflammatory effects against CFA-induced inflammatory pain. The underlying mechanism might involve the activation of α 7nAChR, the inhibition of both HMGB1/NF- κ B signaling and microglial activation, as well as balance between pro-inflammatory cytokine IL-1 β and anti-inflammatory cytokine IL-10 in the spinal cord of CFA rats (Fig. 10). Thus, based on the evidence that ICTF ameliorated CFA-induced pain hypersensitivity through α 7nAChR-mediated inhibition of HMGB1/NF- κ B signaling pathway, which might sever to be a promising therapeutic option for ICTF treating inflammatory pain.

Abbreviations

α 7nAChR: α 7 nicotinic acetylcholine receptor; CFA: complete Freund's adjuvant; DMSO: dimethyl sulfoxide; HE: Hematoxylin eosin; HMGB1: high mobility group box 1; ICTF: Trifluoro-icaritin; i.p.: Intraperitoneal injection; i.t.: Intrathecal injection; PWT: paw withdrawal threshold; PWL: paw withdrawal latency; NSAID: non-steroidal anti-inflammatory drugs.

Declarations

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Credit authors' contributions statement

C.H. and Y.-L.S. Methodology; Y.-L.S. Conceptualization; M.X. Visualization; D.-D.J. G.-S.L. Investigation; Z.-H.H. Project administration; C.H. Writing-Original Draft Writing-Review & Editing. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal experimental procedures strictly followed the guidelines of International Pain Research Association and have been approved by the Animal Protection and Use Committee of Gannan Medical University in P.R. China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

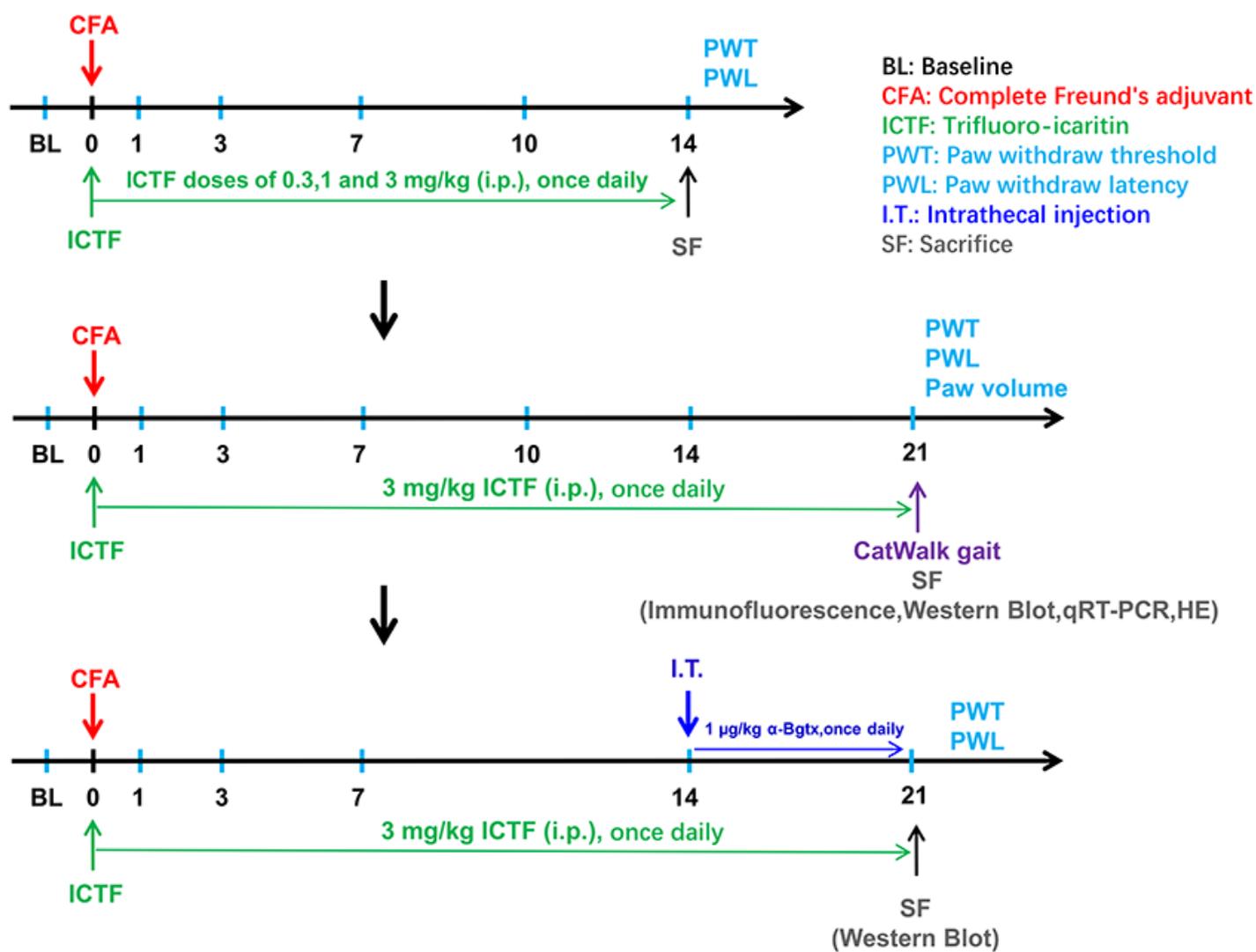


Figure 1

The schematic diagram of experimental procedures.

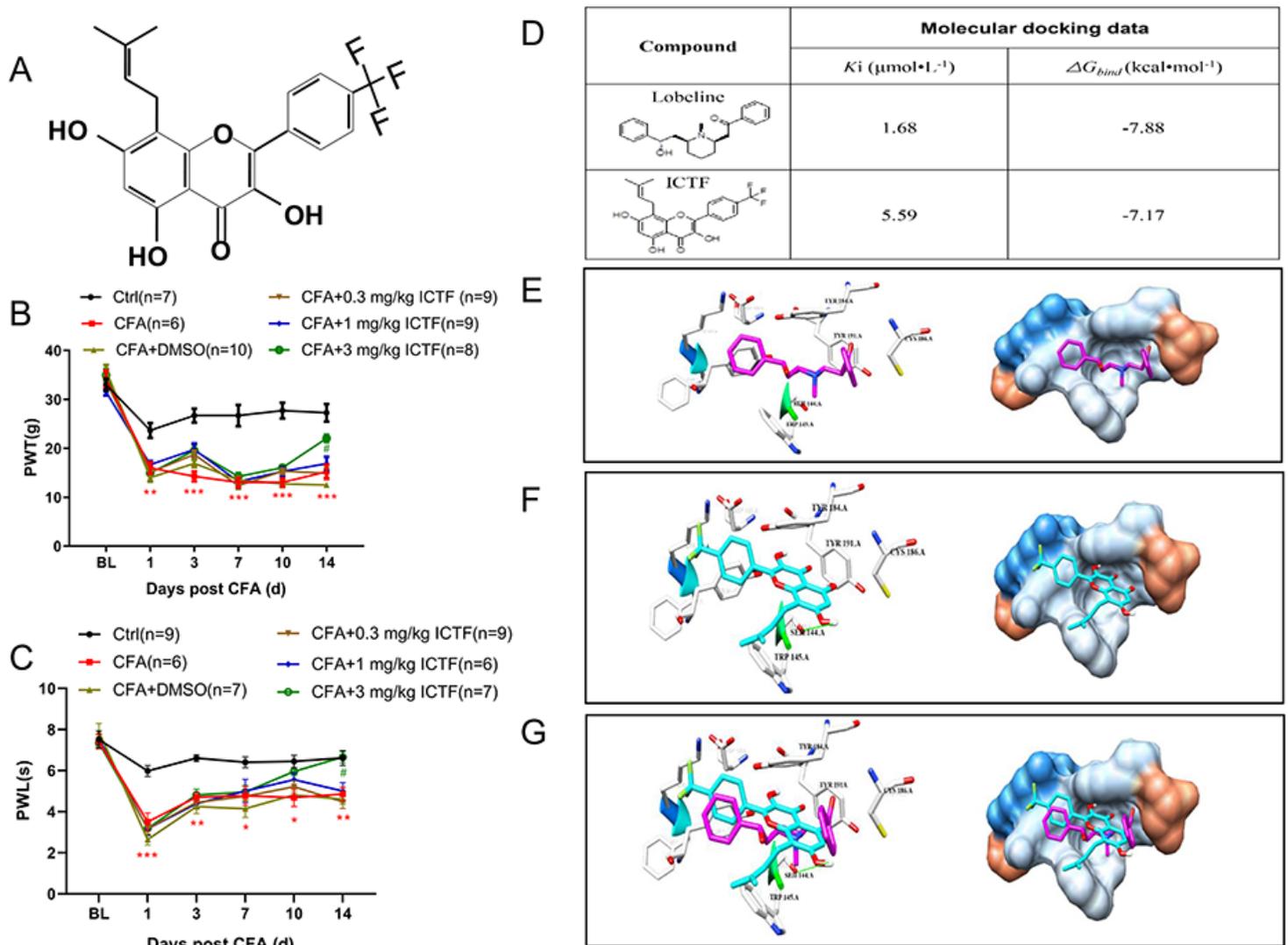


Figure 2

Figure 2

Effect of ICTF treatment on CFA-induced inflammatory pain in rats. (A) Chemical structure of trifluorocaritin (ICTF). (B) CFA-induced mechanical allodynia. (C) CFA-induced thermal hyperalgesia. (D) ΔG is the binding energy, unit: $\text{kcal}\cdot\text{mol}^{-1}$; K_i is the estimated inhibitory constant, unit: $\mu\text{mol}\cdot\text{L}^{-1}$. (E) Lobeline and $\alpha 7\text{nAChR}$ are shown in transparent surface and cartoon representation. (F) ICTF and $\alpha 7\text{nAChR}$ are shown in transparent surface and cartoon representation. (G) Lobeline, ICTF and $\alpha 7\text{nAChR}$ are shown in transparent surface and cartoon representation. CFA rats were treated with the doses of 0.3, 1.0, and 3.0 mg/kg ICTF (i.p.) once daily for 14 days post-CFA injection, the PWT and PWL were detected on day 1 before and day 1, 3, 7, 10, and 14 after CFA injection to evaluate the inhibitory effect of ICTF on CFA rats, respectively. Ctrl represents the Ctrl group; CFA represents rats treated with CFA injection; CFA+DMSO represents rats being treated with CFA and DMSO treatment; CFA + ICTF represents rats receiving CFA injection and ICTF treatment. Data are presented as the mean \pm SEM ($n = 6-10$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with Ctrl group. # $P < 0.05$, compared with CFA group.

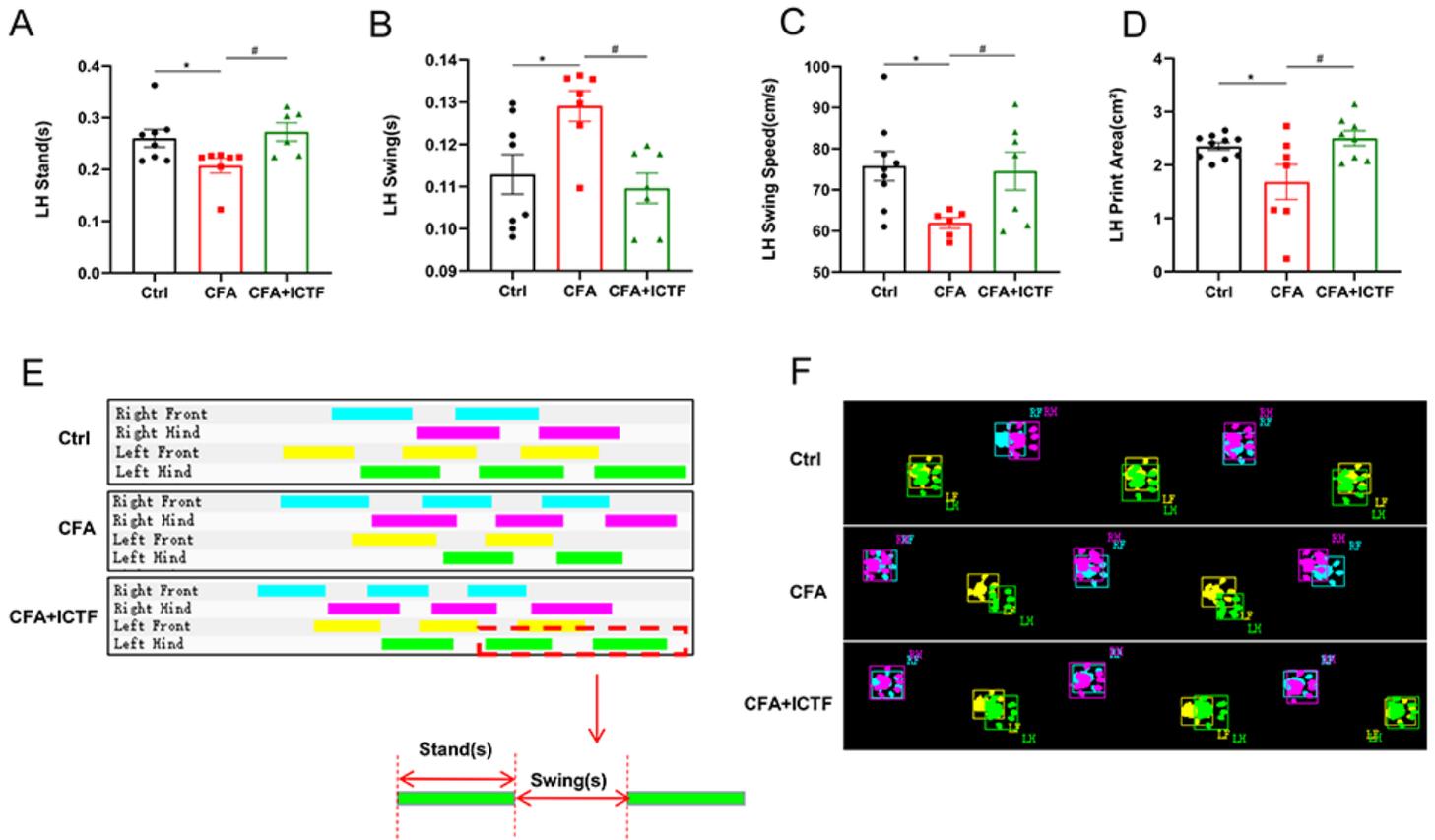


Figure 3

Figure 3

Effect of ICTF on the CatWalk gait parameters in CFA rats. CFA rats were treated with ICTF (3.0 mg/kg, i.p.) once daily for 21 days, then the CatWalk gait parameters were determined on day 21 after CFA. (A) LH Stand(s). (B) LH Swing(s). (C) LH Swing speed(cm/s). (D) LH Print area(cm²). (E) The 2D images of both LH stand and LH swing on day 21 after CFA injection were shown. (F) The visual images of LH print area of rats on day 21 after CFA injection were showed. Ctrl represents the ctrl group; CFA represents rats being treated with CFA injection; CFA+ICTF represents rats receiving CFA and ICTF treatment. Data are presented as the mean \pm SEM (n = 7). *P < 0.05, compared to Ctrl group; #P < 0.05, compared to CFA group.

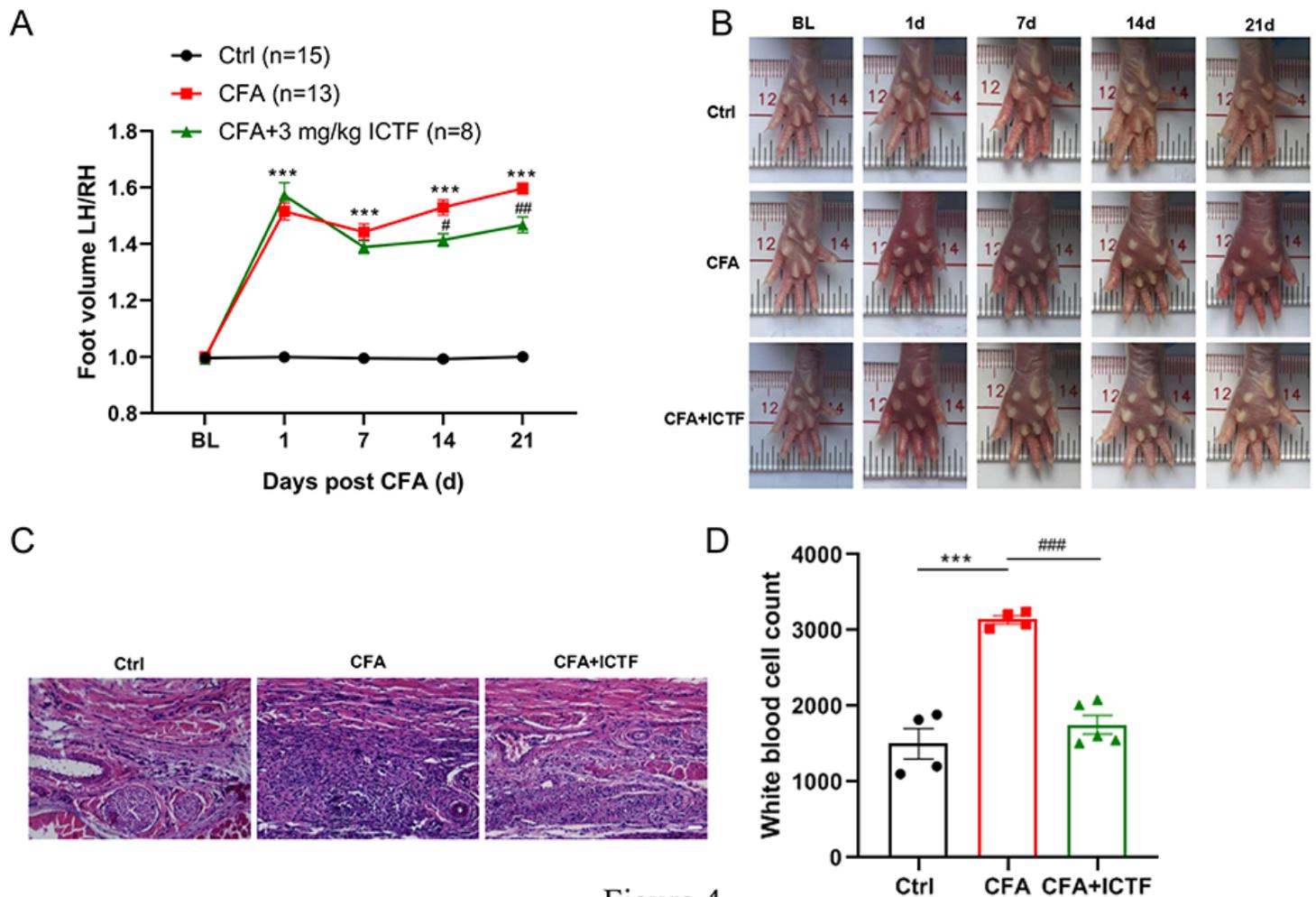


Figure 4

Figure 4

Effect of ICTF on paw tissue inflammation in CFA rats. (A) The time-course effect of ICTF on the paw edema volume (n = 8-15). (B) The representative photographs showed the plantar surface of the hind paw before day 1 and on day 1, 7, 14, and 21 after CFA injection. (C) HE staining of paw tissues in rats was presented. (D) Inflammation of paw tissues with HE staining in rats (n = 4-5). Ctrl represents the ctrl group; CFA represents rats being treated with CFA injection; CFA+ICTF represents rats receiving CFA and ICTF treatment. Data are presented as the mean \pm SEM. ^{***}P < 0.001, compared to Ctrl group; #P < 0.05, ##P < 0.01, ###P < 0.001, compared to CFA group.

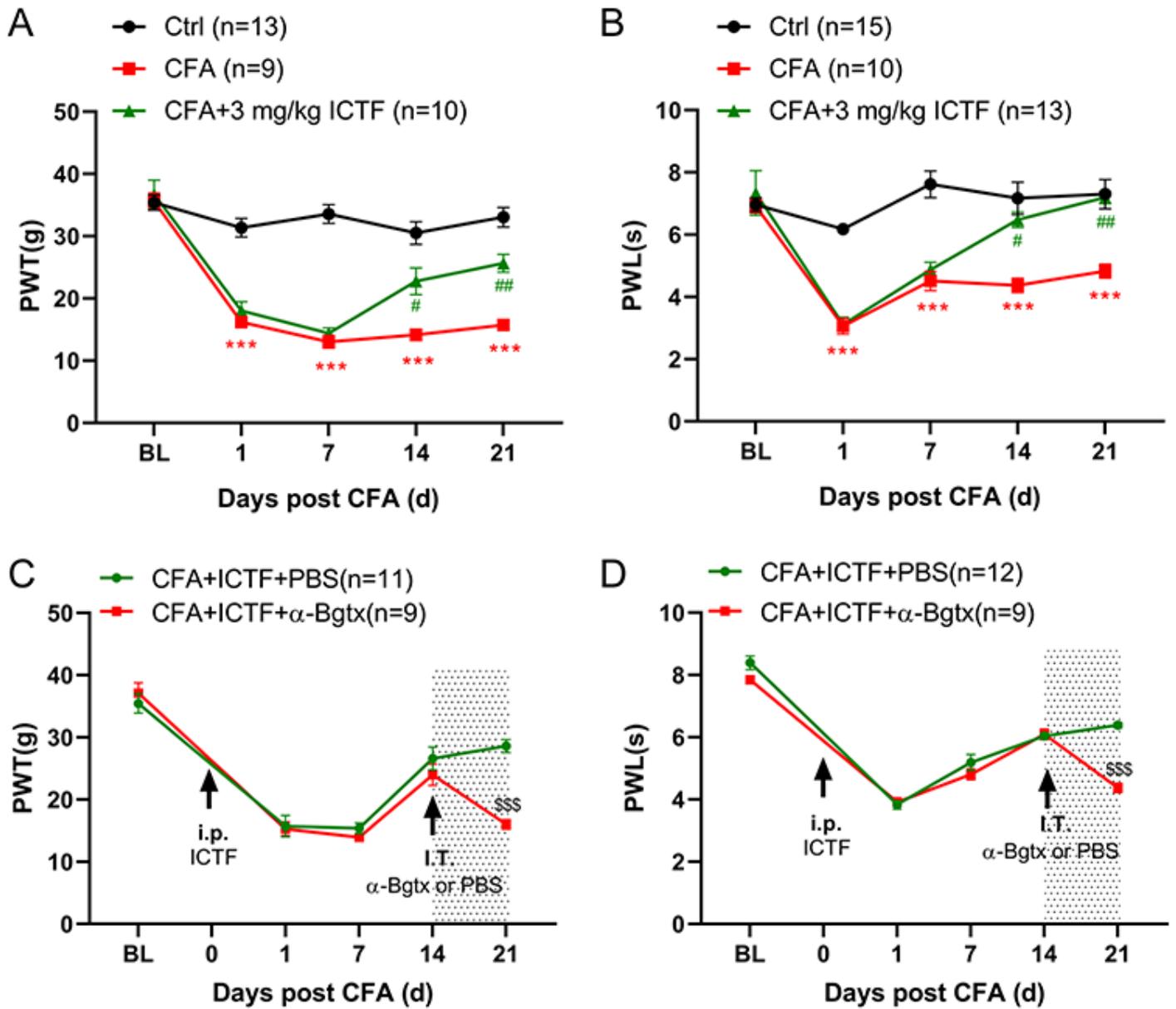


Figure 5

Figure 5

Effect of intrathecal injection of $\alpha 7$ nAChR antagonist α -Bgtx on ICTF-induced anti-nociceptive action in CFA. (A) The time-course effect of ICTF on mechanical allodynia in CFA rats (n=9-13). (B) The time-course effect of ICTF on thermal hyperalgesia in CFA rats (n=10-15). (C) α -Bgtx inhibited the effect of PWT by ICTF in CFA rats (n=9-11). (D) α -Bgtx antagonized the effect of PWL by ICTF in CFA rats (n=9-12). The PWT and PWL were examined on day 7 after intrathecal injection of $\alpha 7$ nAChR antagonist α -Bgtx or PBS, respectively. Data are presented as the mean \pm SEM. ***P < 0.001, compared to Ctrl group, #P < 0.05, ##P < 0.01, compared to CFA group, \$\$\$P < 0.001, compared to CFA+ICTF+PBS group.

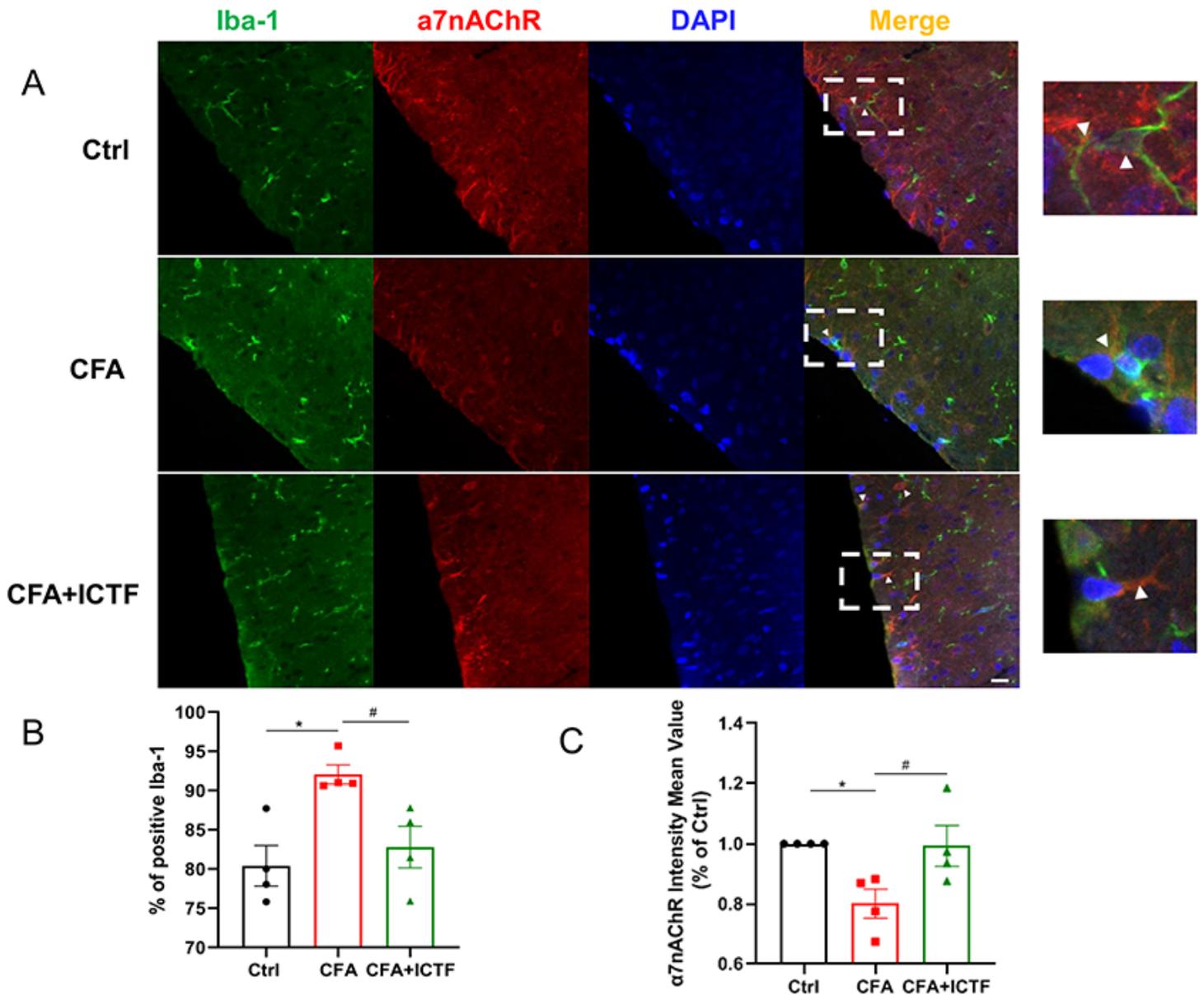


Figure 6

Figure 6

Immunofluorescence staining detected the effect of ICTF on co-expression of spinal α7nAChR with IBA-1 in CFA rats. (A) α7nAChR (red) and microglial activation indicator IBA-1 (green) were double-stained in the spinal dorsal horn from the Ctrl, CFA, and CFA+ICTF group rats (scale bar= 20 μm). (B) Intensity mean value for Iba1. (C) Intensity mean value for α7nAChR. Samples were collected from the L4-L6 segment of spinal cord. Ctrl represents the ctrl group; CFA represents rats being treated with CFA injection; CFA+ICTF represents rats receiving CFA and ICTF treatment. Data are presented as the mean ± SEM (n=4). *P < 0.05, compared to Ctrl group; #P < 0.05, compared to CFA group.

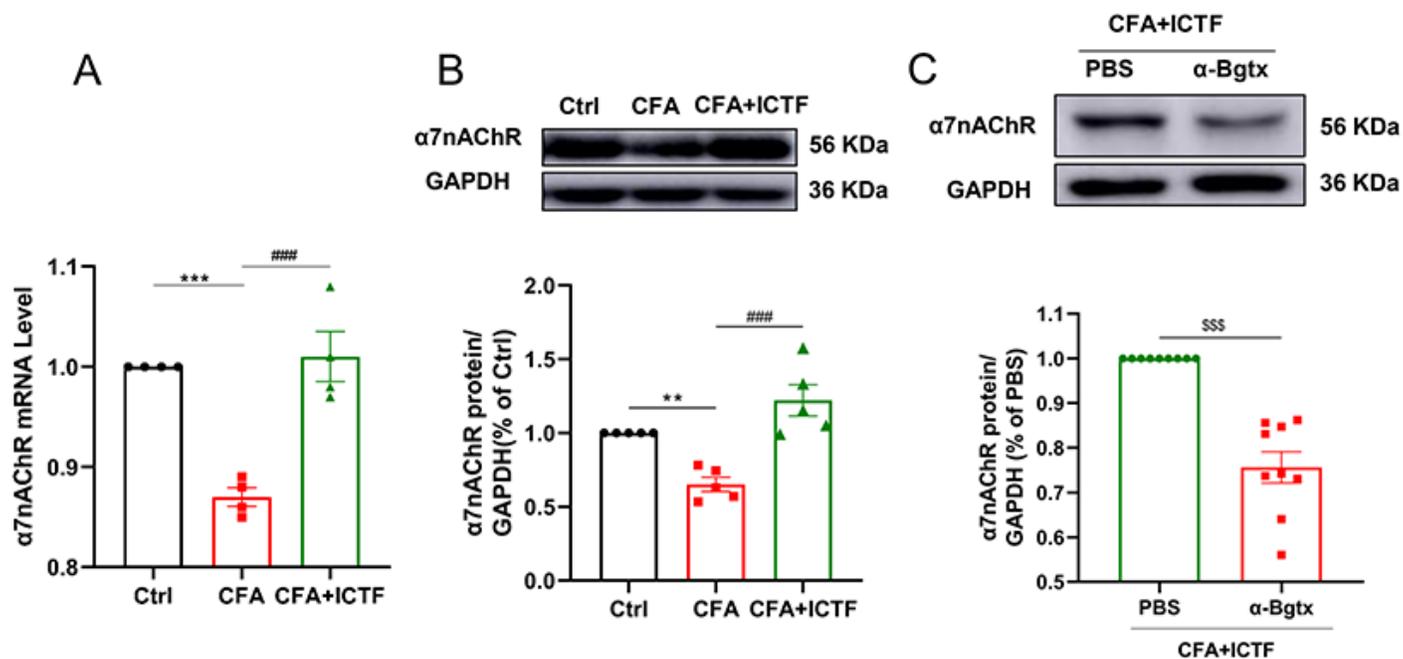


Figure 7

Figure 7

Effect of α -Bgtx on the expression of spinal $\alpha 7nAChR$ induced by ICTF treatment in CFA rats. (A) Relative level of $\alpha 7nAChR$ mRNA expression. (B), (C) Western blotted band and relative level of $\alpha 7nAChR$ protein expression. Samples were collected from the L4-L6 segment of spinal cord. Data are presented as the mean \pm SEM (n = 4-9). **P < 0.01, ***P < 0.001, compared with Ctrl group, ###P < 0.001, compared with CFA group, \$\$\$P < 0.001, compared to CFA+ICTF+PBS group.

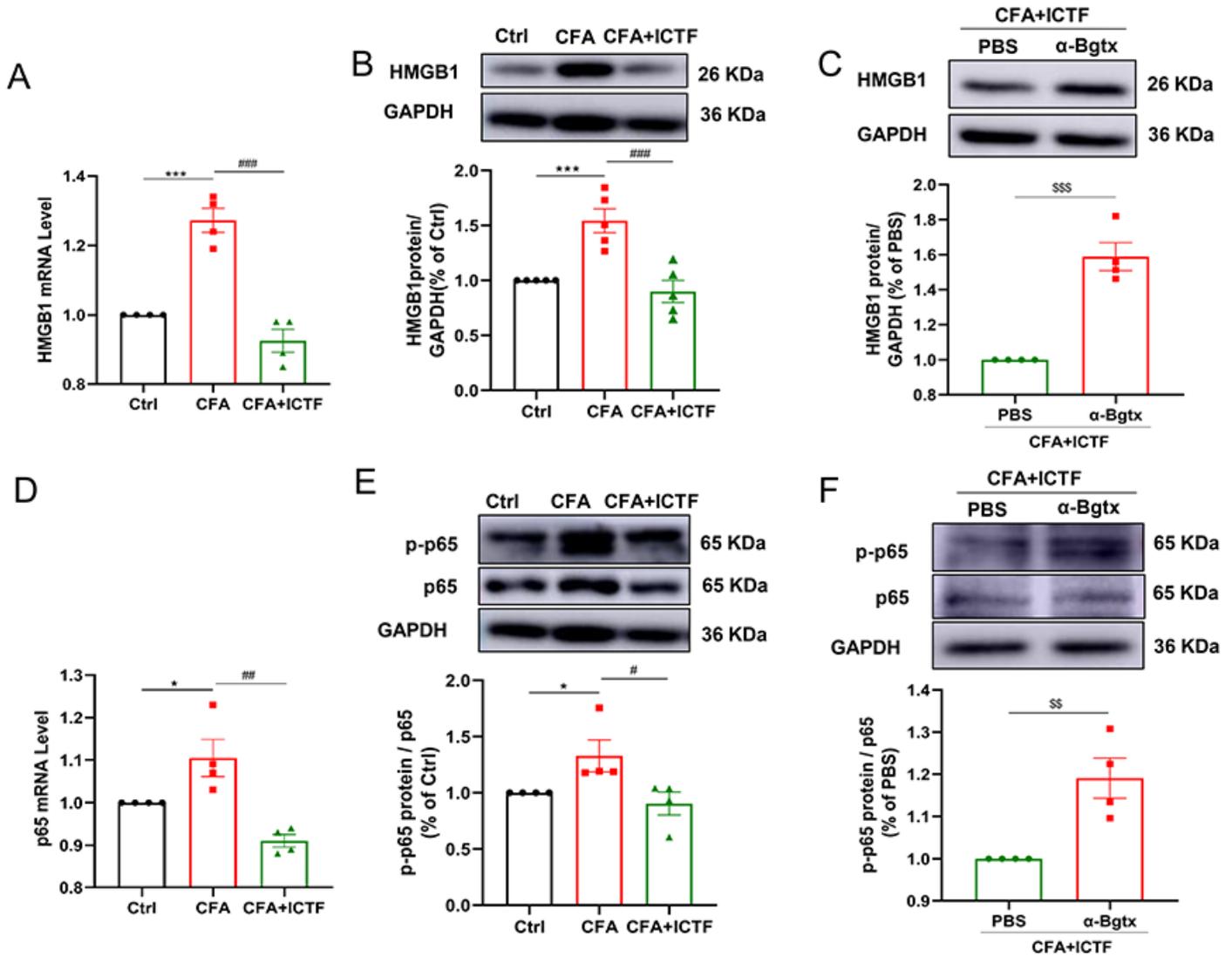


Figure 8

Figure 8

Effect of α -Bgtx on the expression of spinal HMGB1 and NF- κ B p65 induced by ICTF treatment in CFA rats. (A), (D) Relative level of HMGB1 and NF- κ B p65 mRNA expression. (B), (C), (E), (F) Western blotted band and relative level of HMGB1, p-NF- κ B p65 and NF- κ B p65 protein expression. Samples were collected from the L4-L6 segment of spinal cord. Data are presented as the mean \pm SEM (n = 4-5). *P < 0.05, ***P < 0.001, compared with Ctrl group, #P < 0.05, ##P < 0.01, ###P < 0.001, compared with CFA group,

$$P < 0.01,$$

§P < 0.001, compared to CFA+ICTF+PBS group.

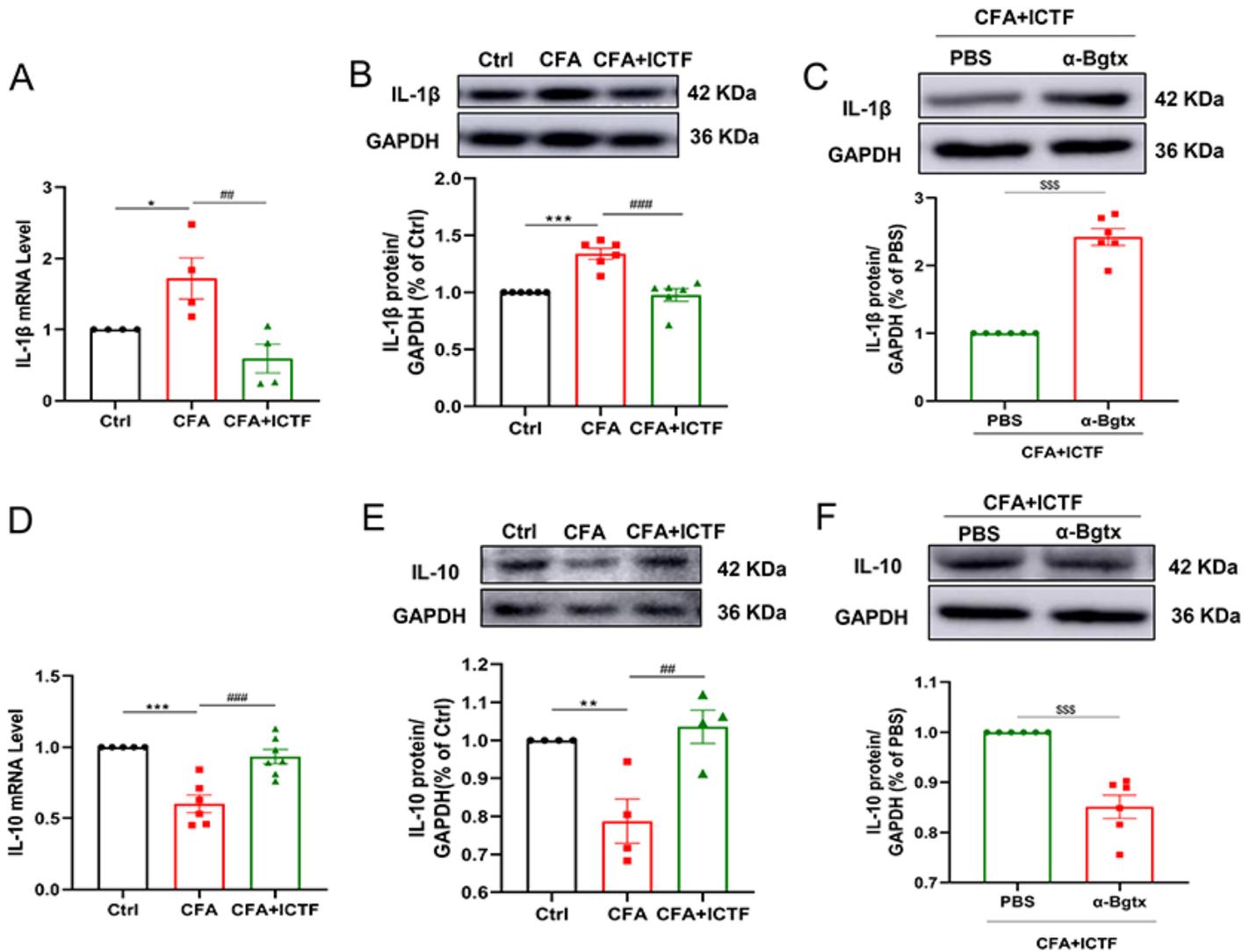


Figure 9

Figure 9

Effect of α -Bgtx on the expression levels of spinal IL-1 β and IL-10 evoked by ICTF treatment in CFA rats. (A), (D) Relative level of IL-1 β and IL-10 mRNA expression. (B), (C), (E), (F) Western blotted band and relative level of IL-1 β and IL-10 protein expression. Samples were collected from the L4-L6 segment of spinal cord. Data are presented as the mean \pm SEM (n = 4-6). *P < 0.05, **P < 0.01, ***P < 0.001, compared with Ctrl group, ##P < 0.01, ###P < 0.001, compared with CFA group, \$\$\$P < 0.001, compared to CFA+ICTF+PBS group.

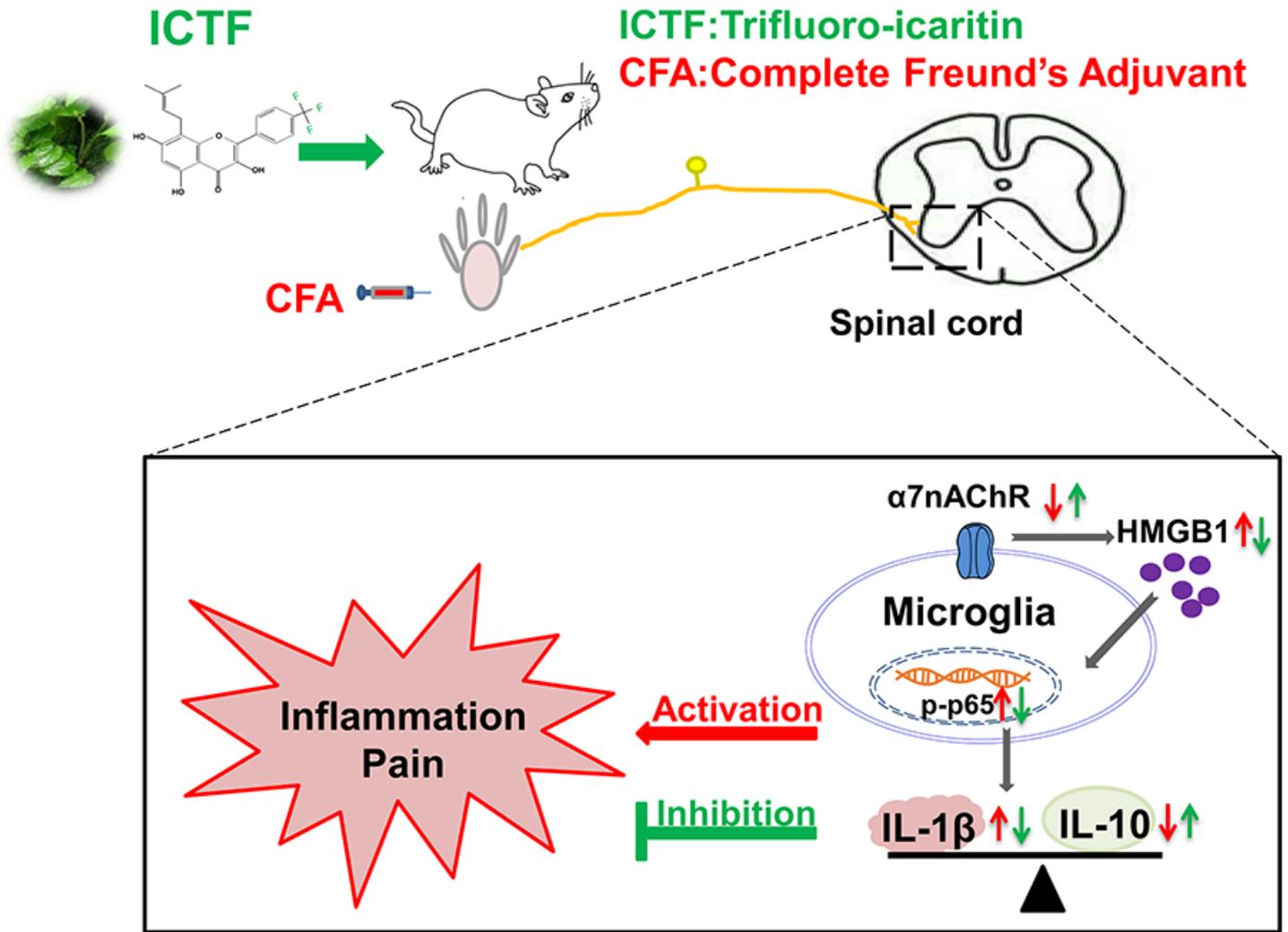


Figure 10

Figure 10

Schematic diagram illustrating the possible mechanisms underlying the anti-inflammatory activity of ICTF on CFA-induced inflammatory pain. Treatment of ICTF to CFA rats effectively attenuated pain-related behaviors, involving mechanical allodynia, thermal hyperalgesia and Catwalk gait parameters through activating $\alpha 7nAChR$ and inhibiting microglial activation, HMGB1/NF- κB signaling pathway activation, as well as balance between pro-inflammatory cytokine and anti-inflammatory cytokine in the spinal cord.