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ORIGINAL RESEARCH

Effects of Combined Shinbaro and Celecoxib in a Complete Freund's Adjuvant-Induced Inflammatory Pain Mouse Model

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Purpose: Persistent inflammation resulting from injury, infection, or arthritis contributes to both peripheral and central sensitization. Various combinations of natural extracts have been explored to minimize the side effects associated with conventional medications. Shinbaro, which has traditionally been used in Eastern medicine to treat inflammatory conditions, was chosen due to its known anti-inflammatory properties. However, previous studies have not yet investigated the combined administration of celecoxib and Shinbaro for their anti-inflammatory and analgesic effects. In this study, we examined the anti-inflammatory and analgesic effects of combining celecoxib with Shinbaro in a complete Freund's adjuvant (CFA)-induced inflammatory pain model.

Methods: We randomly assigned 66 mice to 6 groups (n = 11 per group) and administered intraplantar injections of 100 μL CFA or saline into their right hind paw, followed by oral administration of Shinbaro (100 mg/kg), celecoxib (15 or 30 mg/kg), or both 30 minutes later. Behavioral assessments were conducted blindly at baseline and on days 1, 3, and 7 post-injection. The right hind paw and spinal cord were harvested 3 days post-injection to examine the molecular mechanisms, including macrophage infiltration in the right hind paw, as well as glial cell activation and inflammatory cytokine levels in the spinal cord. Statistical analysis was performed using Tukey's post-hoc test.

Results: The combination of Shinbaro (100 mg/kg) and celecoxib (15 mg/kg) synergistically reduced mechanical hyperalgesia and paw edema by preventing the conversion of monocytes to macrophages and inhibiting macrophage infiltration. Moreover, it decreased the expression of pro-inflammatory cytokines and mediators in the spinal cord by inhibiting spinal microglial activation.

Conclusion: The combination of Shinbaro and celecoxib demonstrates significant anti-inflammatory and analgesic effects, suggesting its potential for managing inflammatory pain with fewer side effects than conventional therapies.

Plain Language Summary: Why was the study done? We wanted to understand if combining two pain-relieving treatments—Shinbaro (an herbal medicine) and celecoxib (a common anti-inflammatory drug)—could work better together than using either one alone. We were particularly interested in treating inflammatory pain, like those experienced in rheumatoid arthritis and osteoarthritis.

What did the researchers do and find? Our team used mice to model inflammatory pain by injecting a substance called CFA into their paw. This caused pain and swelling. We then tested how well Shinbaro and celecoxib worked separately and together to reduce these symptoms. We found that the combination of Shinbaro and celecoxib was more effective at reducing pain and swelling than either treatment alone. The combined treatment decreased the number of inflammatory cells (monocytes and macrophages) in the affected area. In the spinal cord, the combination therapy reduced the activity of cells (microglia and astrocytes) that contribute to ongoing pain. The treatment also lowered the levels of substances that promote inflammation in the body.

What do these results mean? These findings suggest that combining Shinbaro and celecoxib could be a more powerful way to treat inflammatory pain than using either medication by itself. The combination appears to work on multiple levels - reducing inflammation at the site of injury, decreasing pain signals in the spinal cord, and lowering overall inflammation in the body. This approach could potentially allow for lower doses of celecoxib, which might reduce the risk of side effects associated with long-term use.

Keywords: herbal medicine, analgesia, anti-inflammation, natural extracts, paw edema

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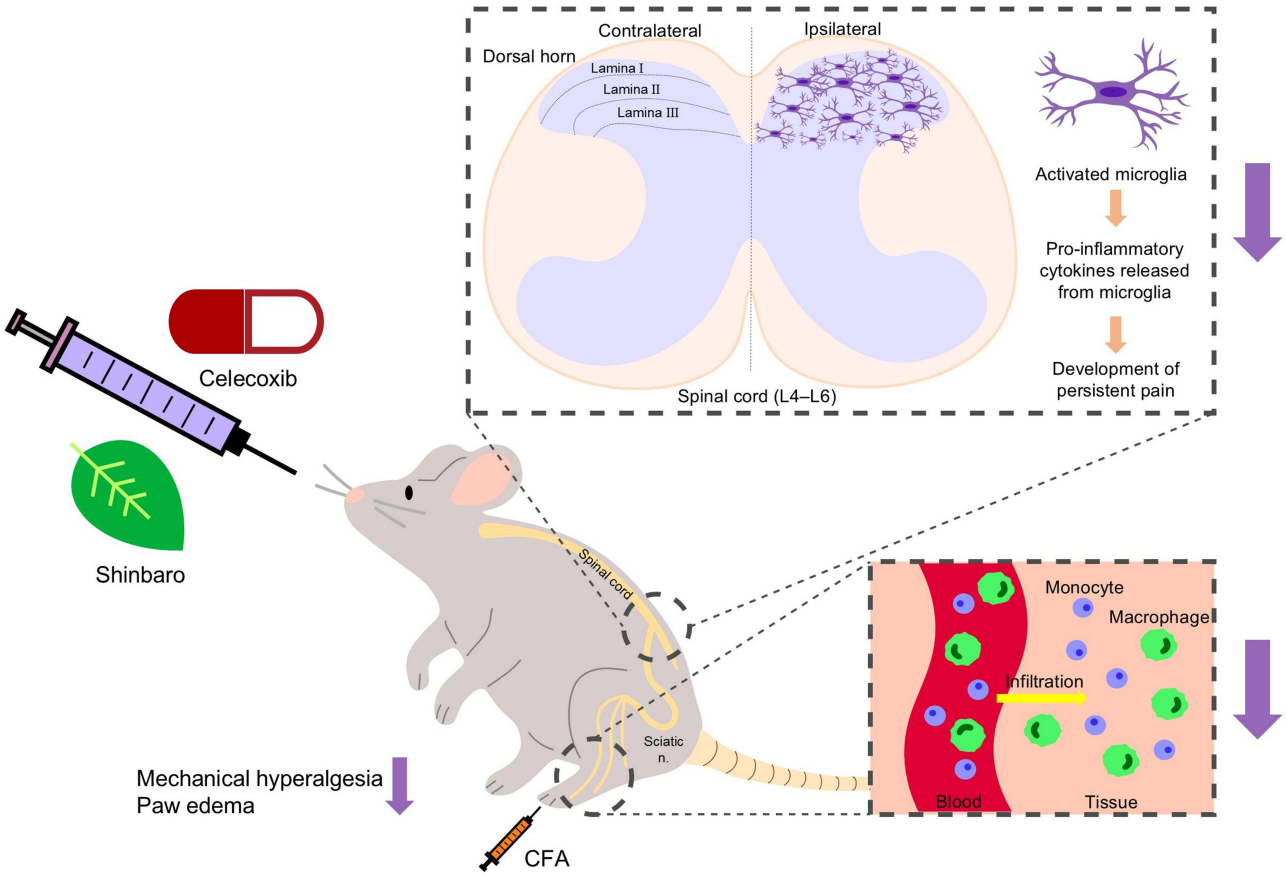
Graphical Abstract



Introduction

Pain caused by central and peripheral sensitization is a key feature of inflammation.[1,2] Inflammatory pain, often leading to chronic pain, is characterized by hyperalgesia, allodynia, and spontaneous pain, making it challenging to manage.[3] The primary treatment strategy for pain associated with inflammation includes non-steroidal anti-inflammatory drugs (NSAIDs).[4] Celecoxib, a representative NSAID and selective cyclooxygenase-2 (COX-2) inhibitor, is commonly prescribed as a first-line analgesic for inflammatory pain such as osteoarthritis and rheumatoid arthritis because of its relatively low risk of gastrointestinal side effects.[5,6] However, long-term NSAID use is linked to cardiovascular disease, anaphylaxis, kidney and liver toxicity, and Stevens–Johnson syndrome.[7,8] Additionally, the cardiovascular risk of celecoxib increases in a dose-dependent manner.[9] Combination therapies offer the potential for synergistic pain relief while reducing adverse side effects at lower celecoxib doses.[10,11]

Shinbaro is a formulation composed of six herbs: *Ledebouriellae Radix, Achyranthis Radix, Acanthopanacis Cortex, Cibotii Rhizoma, Glycine Semen*, and *Eucommiae Cortex*, traditionally used in oriental medicine to treat inflammatory pain, such as knee osteoarthritis.[12] Previous animal studies have shown that intra-articular injections of Shinbaro reduce NF-κB signaling in a rat model of monosodium iodoacetate-induced osteoarthritis, suppressing the expression of pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and pro-inflammatory mediators, including inducible nitric oxide synthase and COX-2.[13] In another study, Shinbaro administered intraperitoneally for 56 days inhibited microglial and astrocyte activation in the dorsal horn of the spinal cord in a rat model of lumbar disc

herniation.[14] Moreover, in a pilot study on patients with migraine, a 12-week Shinbaro treatment reduced migraine frequency and calcitonin gene-related peptide levels in the blood.[15]

The combination of celecoxib and other medications, using their distinct pharmacokinetic and pharmacodynamic interactions, may provide effective pain relief for various conditions and reduce side effects by lowering the required dosage of celecoxib.[10,16–18] However, studies investigating the combination therapy of Shinbaro and celecoxib for treating inflammatory pain have not yet been reported. Therefore, we investigated whether the combination of Shinbaro and celecoxib had a synergistic effect in alleviating inflammatory pain in a mouse model of complete Freund's adjuvant (CFA)-induced inflammatory pain. By evaluating this combination therapy, we sought to gain insights into a safer and more effective pain management strategy, potentially reducing the dosage of celecoxib needed and minimizing associated risks.

Materials and Methods

Animals

Eight-week-old male C57BL/6 mice (weight, 22–25 g; DBL Co. Ltd, Eumseong, Korea) were randomly assigned to groups and housed at 22 ± 2°C under a 12-hour light/dark cycle with free access to food and water for ≥7 days prior to the experiments. All procedures followed institutional guidelines and regulations for the care and use of laboratory animals and were approved by the Jaseng Animal Care and Use Committee [IACUC No. JSR-2024-01-003-A].

Inflammatory Pain Model

A CFA oil suspension diluted in saline (1:1) was used to induce chronic inflammatory pain. Mice were anesthetized with 2–3% isoflurane (Forane; BK Pharm, Goyang, Republic of Korea) before intraplantar (i.pl). injection of the CFA (100 μL; Sigma, St. Louis, MO, USA) emulsion into the right hind paw.[19] Mice in the Normal group (control group for CFA injection) were injected with saline (100 μL) into the right hind paw.

Drug Administration

Celecoxib is effective at doses ranging from 10 to 50 mg/kg.[20] Celecoxib (15 and 30 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a solution of 0.5% carboxymethyl cellulose (CMC) sodium (Sigma-Aldrich) in distilled water. Shinbaro (GCSB-5; Green Cross Corp., Yongin, Korea) is an industrial product manufactured and produced according to the contents, method of extraction, and preparation of the final formulation reported in a previous research.[12] In this study, the concentration of Shinbaro (100 mg/kg) was used based on a previous in vivo study.[21] Shinbaro was ground and dissolved in a 0.5% CMC solution. Shinbaro (100 mg/kg) and celecoxib (15 mg/kg) were then administered in combination. Mice received 100 μL of the designated treatment solution orally (p.o). once daily for 3 or 7 days, beginning 30 minutes after the CFA injection (Day 0). Subsequently, 66 mice were divided into 6 groups (n = 11 per group): Normal group (0.5% CMC with saline), CFA group (0.5% CMC with CFA), CFA+Shin group (Shinbaro [100 mg/kg] with CFA), CFA+Cel15 group (celecoxib [15 mg/kg] with CFA), CFA+Cel30 group (celecoxib [30 mg/kg] with CFA), and CFA+Shin+Cel15 group (Shinbaro [100 mg/kg] + celecoxib [15 mg/kg] with CFA).

Behavior Tests

Mechanical hyperalgesia in both hind paws was evaluated using the von Frey test (Dynamic Plantar Aesthesiometer; Ugo Basile, Varese, Italy). Progressively increasing filament forces (0–5.0 g) were applied to the plantar surface of each hind paw (five times for 10 seconds). The latency time and paw withdrawal threshold for filament removal from the plantar surface of the hind paw were measured, and five measurements were averaged. The von Frey test was performed on days 1, 3, and 7 after CFA injection.

Improvement rate of latency time (%) = [(latency time of the drug administration group at 7 days – latency time of the CFA group at 7 days)/ latency time of the CFA group at 7 days] × 100

Improvement rate of paw withdrawal threshold (%) = [(paw withdrawal threshold of the drug administration group at 7 days – paw withdrawal threshold of the CFA group at 7 days)/ paw withdrawal threshold of the CFA group at 7 days] × 100

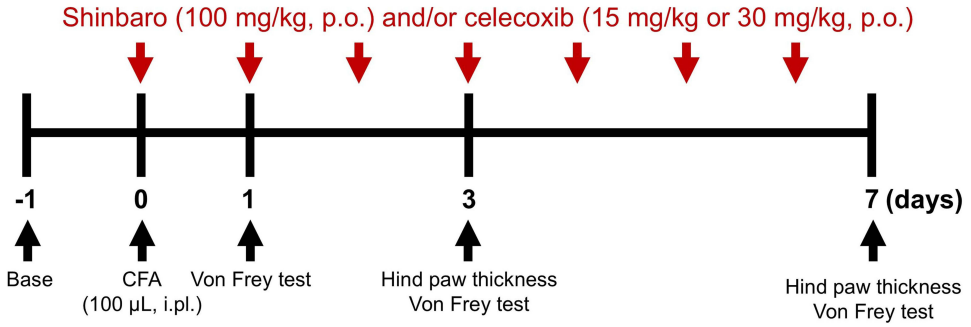


Figure 1 Experimental process for combining Shinbaro and celecoxib in a complete Freund's adjuvant-induced inflammatory pain model. CFA (100 μL) was injected intraplantarly (i.pl) into the right hind paw of mice, and Shinbaro (100 mg/kg), celecoxib (15 or 30 mg/kg), or both, were administered orally (P.O.) after 30 min. The drug was administered daily for 3 or 7 days. Behavioral tests were conducted at baseline 1 day before the CFA injection and then on days 1, 3, and 7 after the CFA or saline (100 μL) injection.

To observe the effects on paw edema, the right hind paw thickness was measured using a digital caliper (Advanced Onsite Sensor Absolute Scale Digital Caliper; Mitutoyo, Kawasaki, Japan) on days 3 and 7 after CFA injection. Both mechanical hyperalgesia and hind paw thickness were measured at baseline 1 day before the initial CFA injection. The experimental design is illustrated in Figure 1. All behavioral tests were conducted in a blind manner by different evaluators and analysts.

Improvement rate of hind paw thickness (%) = [(hind paw thickness of the drug administration group at 7 days – hind paw thickness of the CFA group at 7 days)/ hind paw thickness of the CFA group at 7 days] × 100

Fluorescence-Activated Cell Sorting (FACS)

The right hind paw was amputated from the mice 3 days after the initial CFA injection, washed in phosphate-buffered saline (PBS), and the toes and bones were removed. Tissues were incubated at 37°C for 3 hours in Roswell Park Memorial Institute (RPMI) 1640 medium containing 100 U/mL of Dispase II (Gibco), 10 mg/mL of Collagenase IV, and 10 mg/mL of DNase I. Next, the tissues were homogenized in RPMI 1640 medium containing Dispase II, Collagenase IV, and DNase I and filtered through a cell strainer (40 μm). To isolate leukocytes from the tissues, centrifugation was performed using an RBC Lysis Buffer (eBioscience) and 35% v/v Percoll (Sigma-Aldrich). Extracted cells were stained with antibodies at 4°C for 30 min.[22] The following antibodies were used: CD3-FITC (T cells marker, #555274; BD Biosciences), F4/80-Per-CP (macrophage marker, #567202; BD Biosciences), and CD11b-APC (monocyte marker, #553312; BD Biosciences). An Fc receptor-blocking step with Purified Rat Anti-Mouse CD16/32 (#553142; BD Biosciences) was performed to minimize the nonspecific binding of antibodies. Samples were acquired and analyzed using an Accuri C6 Plus Flow Cytometer (BD Biosciences).

Immunofluorescence

Spinal cords were extracted from mice 3 days after the initial CFA injection and post-fixed in 4% formaldehyde at 4°C. The spinal cord was sectioned at 40 μm using a cryostat microtome (Leica CM 1520, Leica Biosystems, Nussloch, Germany). Immunofluorescence analysis was performed,[23] to evaluate the expression of ionized calcium-binding adaptor molecule 1 (Iba-1; microglia marker), glial fibrillary acidic protein (GFAP; astrocyte marker), and COX-2 in the L4–L6 dorsal horn of the spinal cord. Primary antibodies against Iba-1 (rabbit, 1:100, #17198; Cell Signaling Technology, Beverly, MA, USA), GFAP (mouse, 1:1000, 14–9892-82; Invitrogen, Carlsbad, CA, USA), and COX-2 (rabbit, 1:500, #12282S; Cell Signaling Technology) were diluted in 1× PBS-T (PBS with 0.3% Triton X-100) supplemented with 0.5% goat serum. The tissue slides were wrapped to block light and stored at 4°C for 72 hours. The tissues were then sequentially incubated for 1 hour with a mixture of donkey anti-rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (A21206; Invitrogen), and donkey anti-mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 (A21203; Invitrogen). Optical density was measured using ImageJ (Java 1.8.0_172, National Institutes of Health, USA) in a square grid (100 × 100 μm) in laminae I and II of the spinal cord dorsal horn.

Enzyme-Linked Immunosorbent Assays (ELISA)

The spinal cord at L4–L6 was extracted from mice 3 days after the initial CFA injection and homogenized with RIPA lysis buffer containing 1% PMSF (Solarbio, China) to extract the total protein for ELISA of inflammatory cytokines.[24] The contents of pro-inflammatory cytokines were measured using a mouse TNF-α high-sensitivity ELISA kit (LABISKOMA, #K0331230HS), mouse IL-6 high-sensitivity ELISA kit (LABISKOMA, #K0331186HS), and mouse IL-1β/IL-1F2 ELISA kit (R&D systems, #MLB00C-1). All protocols were performed according to the manufacturer's instructions. Absorbance was measured at 450 nm using an Epoch microplate reader (BioTek, Winooski, VT, USA).

Statistical Analysis

All data are presented as the mean ± standard deviation, and statistical analyses were performed using GraphPad Prism 8.0.1 software (GraphPad Software, San Diego, CA, USA). Data were analyzed using one-way and two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. In all analyses, statistical significance was set at $p < 0.05$.

Results

Combination of Shinbaro and Celecoxib Has a Synergistic Effect on Pain Relief in a CFA-Induced Inflammatory Pain Model

To investigate the analgesic effect of combined Shinbaro and celecoxib, we assessed mechanical hyperalgesia using the von Frey test in a CFA-induced inflammatory pain model. Two-way ANOVA revealed significant latency time ($F_{3,90} = 31.17$, $p < 0.0001$) and interaction ($F_{15, 90} = 3.817$, $p < 0.0001$). Tukey's post-hoc test showed that the CFA group experienced a significant decrease starting from day 3 (day 3, $p = 0.0007$; day 7, $p < 0.0001$ vs Normal). The CFA+Cel15 (day 3, $p = 0.0042$; day 7, $p = 0.0628$), CFA+Cel30 (day 3, $p = 0.0004$; day 7, $p = 0.0012$), and CFA + Shin +Cel15 (day 3, $p = 0.0003$; day 7, $p = 0.0004$) groups showed significant increases in latency time compared to the CFA group (Figure 2A and Supplementary Table 1). On day 7, one-way ANOVA indicated a significant difference in latency time among the groups ($F_{5,30} = 11.51$, $p < 0.0001$). Tukey's post-hoc test revealed that latency time was higher in the CFA +Shin+Cel15 group than in the CFA+Shin ($p = 0.0217$), CFA+Cel15 ($p = 0.0124$), and CFA+Cel30 groups ($p = 0.2322$; Figure 2B). The improvement in latency time was greater in the CFA+Shin+Cel15 group than in the CFA+Shin, CFA +Cel15, and CFA+Cel30 groups (Table 1).

Similarly, for the paw withdrawal threshold, two-way ANOVA revealed a significant latency time ($F_{3,90} = 30.68$, $p < 0.0001$) and a significant interaction ($F_{15,90} = 3.651$, $p < 0.0001$). Tukey's post-hoc test indicated that the CFA group experienced a decrease in withdrawal threshold compared with the Normal group on days 3 ($p = 0.0022$) and 7 ($p < 0.0001$), whereas the CFA+Cel15 (day 3, $p = 0.0676$; day 7, $p = 0.1533$), CFA+Cel30 (day 3, $p = 0.0125$; day 7, $p = 0.0125$), and CFA+Shin+Cel15 (day 3, $p = 0.0008$; day 7, $p = 0.0004$) groups experienced an increase in paw withdrawal threshold compared to the CFA group (Figure 2C and Supplementary Table 2). On day 7, one-way ANOVA revealed a significant difference in paw withdrawal threshold among the groups ($F_{5,30} = 11.55$, $p < 0.0001$). Tukey's post-hoc test revealed that the paw withdrawal threshold was higher in the CFA+Shin+Cel15 group than in the CFA+Shin ($p = 0.0169$), CFA+Cel15 ($p = 0.0108$), and CFA+Cel30 groups ($p = 0.1930$; Figure 2D). The CFA+Shin+Cel15 group showed a greater improvement in paw withdrawal threshold compared to the CFA+Shin, CFA+Cel15, and CFA+Cel30 groups (Table 2). These results suggest that the combination of Shinbaro and celecoxib synergistically improved mechanical hyperalgesia in a CFA-induced inflammatory pain model.

Combination of Shinbaro and Celecoxib Has a Synergistic Effect on Reducing Hind Paw Inflammation

To examine whether combination therapy with Shinbaro and celecoxib could suppress hind paw inflammation, hind paw thickness was measured. Two-way ANOVA revealed a significant latency time ($F_{2,60} = 284.7$, $p < 0.0001$) and interaction ($F_{10,60} = 14.63$, $p < 0.0001$) for hind paw thickness. Tukey's post-hoc test showed that hind paw thickness significantly increased three days after CFA injection (day 3, $p < 0.0001$; day 7, $p < 0.0001$ vs Normal) and significantly improved in the CFA+Shin+Cel15 group (day 3, $p < 0.0001$; day 7, $p < 0.0001$) compared with the CFA group

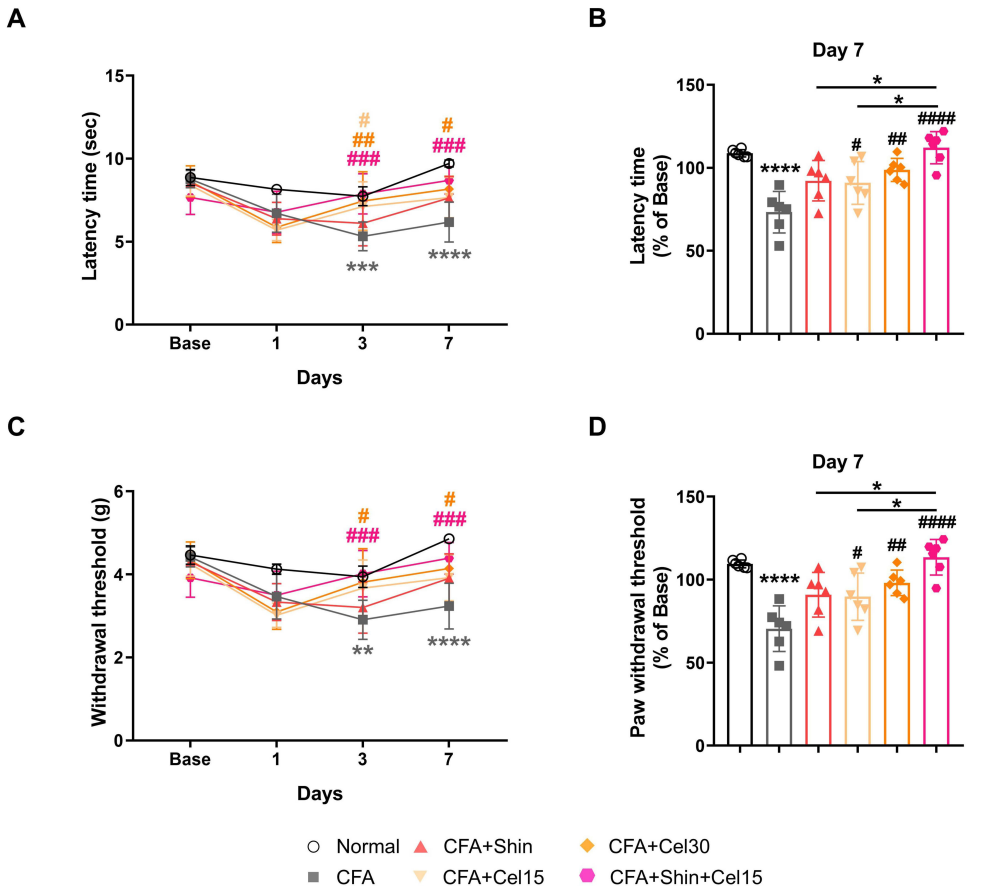


Figure 2 Effect of combined Shinbaro and celecoxib treatment on mechanical hyperalgesia induced by CFA. (**A** and **B**) Latency time, which assessed the time to withdrawal in response to gradually increasing filament pressure (**C** and **D**), and paw withdrawal threshold, which assessed the filament pressure at the time of withdrawal, were measured using the von Frey test 1, 3, and 7 days after CFA or saline injection. n = 6/group. *$p < 0.05$ vs comparative group, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs Normal, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs CFA. Results were analyzed using one-way and two-way analysis of variance followed by Tukey's post-hoc test. The results are presented as mean ± standard deviation.

(Figure 3A and B, and Supplementary Table 3). One-way ANOVA revealed a significant difference in hind paw thickness among the groups on the seventh day ($F_{5,30} = 19.62$, $p < 0.0001$) after CFA injection. Tukey's post-hoc test indicated that hind paw thickness was lower in the CFA+Shin+Cel15 group than in the CFA+Shin ($p = 0.0286$), CFA+Cel15 ($p = 0.0054$), and CFA+Cel30 ($p = 0.0280$) groups (Figure 3C). The improvement rate in hind paw thickness was greater in the CFA+Shin+Cel15 group than in the CFA+Shin, CFA+Cel15, and CFA+Cel30 groups combined (Table 3). These findings suggest that the combination of Shinbaro and celecoxib synergistically suppressed hind paw inflammation in the CFA-induced inflammatory pain model.

Table 1 Improvement Rate of Latency Time on the Combination Therapy of Shinbaro and Celecoxib for 7 days

Improvement Rate of Latency Time (%)	
CFA+Shinbaro	25.30%
CFA+celecoxib (15mg/kg)	29.68%
CFA+celecoxib (30 mg/kg)	36.81%
CFA+Shinbaro+celecoxib (15 mg/kg)	61.09%

Notes: vs CFA.
Abbreviation: CFA, complete Freund's adjuvant.

Table 2 Improvement Rate of Paw Withdrawal Threshold on the Combined Administration of Shinbaro and Celecoxib for 7 days

Improvement Rate of Paw Withdrawal Threshold (%)	
CFA+Shinbaro	22.69%
CFA+celecoxib (15mg/kg)	26.06%
CFA+celecoxib (30 mg/kg)	32.72%
CFA+Shinbaro+celecoxib (15 mg/kg)	53.06%

Notes: vs CFA.

Combination Therapy of Shinbaro and Celecoxib Attenuated the Infiltration of Macrophages in the Hind Paw

To observe the effects of combined Shinbaro and celecoxib on the infiltration of macrophages and monocytes into tissues, FACS was conducted on hind paw tissues 3 days after CFA or saline injection. One-way ANOVA showed a significant difference among the groups in the number of $CD3^+F4/80^+$ cells ($F5,24 = 19.27$, $p < 0.0001$) and $CD3^+CD11b^+$ cells ($F5, 24 = 46.10$, $p < 0.0001$). Tukey's post-hoc test revealed that the number of $CD3^+F4/80^+$ ($p < 0.0001$) and $CD3^+CD11b^+$ ($p < 0.0001$) cells was higher in the CFA group than in the Normal group. Additionally, the number of $CD3^+F4/80^+$ cells was lower in the CFA+Shin+Cel15 group than in the CFA group ($p = 0.0394$; Figure 4A and D); however, there was no difference in the number of $CD3^+CD11b^+$ cells (Figure 4B and E). Furthermore, one-way ANOVA revealed a significant difference in the number of F4/80+CD11b+ cells ($F5,24 = 44.18$, $p < 0.0001$). Tukey's post-hoc test indicated that the number of $F4/80^+CD11b^+$ cells decreased in the CFA+Shin+Cel15 group compared to that in the CFA group ($p = 0.0349$; Figure 4C and F). These results suggest that the combination of Shinbaro and celecoxib more effectively reduced CFA-induced macrophage infiltration into tissues than either agent alone.

Combination Therapy of Shinbaro and Celecoxib Inhibited the Activation of Microglia and Astrocytes in the Spinal Cord Dorsal Horn

Intraplantar injection of CFA increases glial cell activation and inflammatory cytokine levels in the spinal cord dorsal horn, as well as inflammation in the footpad. These results may lead to inflammatory pain, which can develop into chronic increased pain sensitivity.[25–28] To investigate the effects of the combined Shinbaro and celecoxib treatment on the activation of microglia and astrocytes in the dorsal horn of the spinal cord in a CFA-induced inflammatory pain

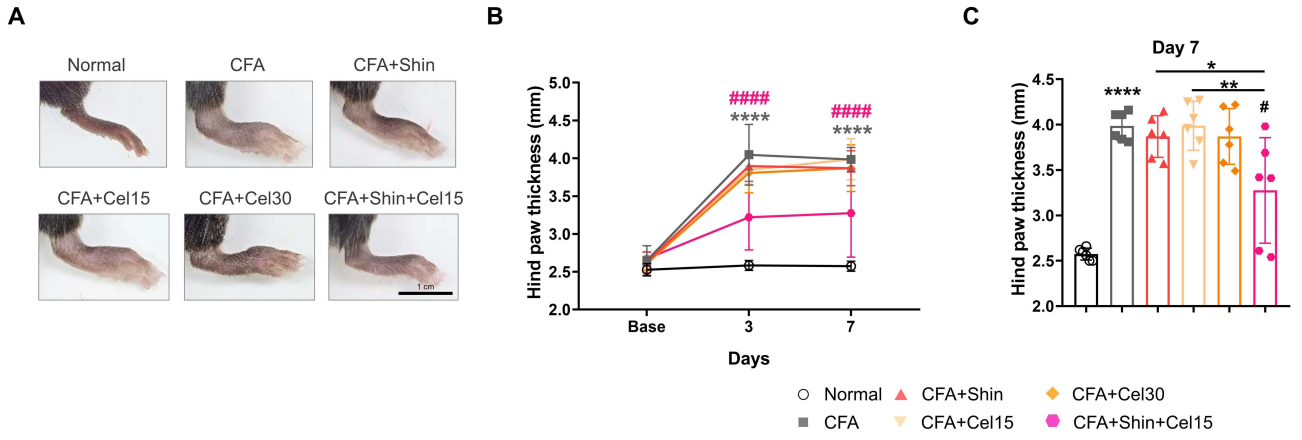


Figure 3 Effect of combined Shinbaro and celecoxib treatment on hind paw edema induced by CFA. (**A**–**C**) To evaluate hind paw edema, hind paw thickness was measured using a digital caliper 3 and 7 days after the CFA or saline injection. Scale bar: 1 cm. n = 6/group. *$p < 0.05$, **$p < 0.01$ vs comparative group, ****$p < 0.0001$ vs Normal, $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ vs CFA. Data were analyzed using one-way and two-way analysis of variance, followed by Tukey's post-hoc test. All data are expressed as mean ± standard deviation.

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Table 3 Improvement Rate of Hind Paw Thickness on the Combination of Shinbaro and Celecoxib for 7 days

Improvement Rate of Hind Paw Thickness (%)	
CFA+Shinbaro	2.93%
CFA+celecoxib (15mg/kg)	−0.08%
CFA+celecoxib (30 mg/kg)	2.89%
CFA+Shinbaro+celecoxib (15 mg/kg)	17.82%

Notes: vs CFA.

model, we assessed the expression of Iba-1 and GFAP in the ipsilateral dorsal horn of the lumbar spinal cord (L4–L6) using immunofluorescence staining (Figure 5A). One-way ANOVA revealed significant differences among the groups in the expression of Iba-1 ($F5,24 = 5.039$, $p = 0.0027$) and GFAP ($F5, 24 = 8.147$, $p = 0.0001$). Tukey's post-hoc test indicated that Iba-1 expression was higher in the CFA group than in the Normal group ($p = 0.0024$) and was significantly reduced in the CFA+Shin+Cel15 group compared to the CFA group ($p = 0.0036$). The CFA+Cel30 group was not significantly different from the CFA group ($p = 0.2099$; Figure 5B). In addition, GFAP expression increased in the CFA group compared to the Normal group ($p = 0.0005$) but did not show a significant decrease due to drug administration (Figure 5C). These results suggest that the combination of Shinbaro and celecoxib attenuates the activation of microglia in the dorsal horn of the spinal cord.

Combination Therapy of Shinbaro and Celecoxib Reduced Expression of Pro-Inflammatory Cytokines in the Spinal Cord

To examine the effect of the combination of Shinbaro and celecoxib on pro-inflammatory cytokines in the spinal cord, we measured the expression of TNF-α, IL-6, and IL-1β in the L4–L6 spinal cord using ELISA. One-way ANOVA showed significant differences among the groups for TNF-α expression ($F5,24 = 7.856$, $p = 0.0002$), IL-6 ($F5,24 = 5.157$, $p = 0.0024$), and IL-1β ($F5,24 = 3.818$, $p = 0.0110$). Tukey's post-hoc test revealed that TNF-α expression was higher in the CFA group than in the Normal group ($p = 0.0002$). Compared to the CFA group, TNF-α expression was significantly reduced in the CFA+Shin ($p = 0.0152$), CFA+Cel30 ($p = 0.0102$), and CFA+Shin+Cel15 ($p = 0.0020$) groups (Figure 6A). IL-6 expression was significantly higher in the CFA group than in the Normal group ($p = 0.0019$) and was significantly lower in the CFA+Shin+Cel15 group than in the CFA group ($p = 0.0064$). There was no significant decrease in the CFA+Cel30 group compared to the CFA group ($p = 0.1168$; Figure 6B). Additionally, IL-1β expression was significantly higher in the CFA group than in the Normal group ($p = 0.0212$), but no significant reduction was observed in the drug-treated groups (Shinbaro and/or celecoxib) compared to the CFA group (Figure 6C).

Combination Therapy of Shinbaro and Celecoxib Decreased the Expression of Pro-Inflammatory Mediators in the Dorsal Horn of the Spinal Cord

To investigate the effect of the combination of Shinbaro and celecoxib on COX-2 expression, we analyzed its expression in the dorsal horn of the spinal cord. One-way ANOVA indicated a significant difference in COX-2 expression among the groups ($F5,24 = 4.415$, $p = 0.0054$). Tukey's post-hoc test showed that COX-2 expression was higher in the CFA group than in the Normal group ($p = 0.0385$) and significantly lower in the CFA+Shin+Cel15 group than in the CFA group ($p = 0.0097$). The CFA+Cel30 group showed no significant decrease compared to the CFA group ($p = 0.5643$; Figure 7A and B). These findings suggest that the combination of Shinbaro and celecoxib suppresses the CFA-induced expression of pro-inflammatory cytokines and mediators in the L4–L6 spinal cord.

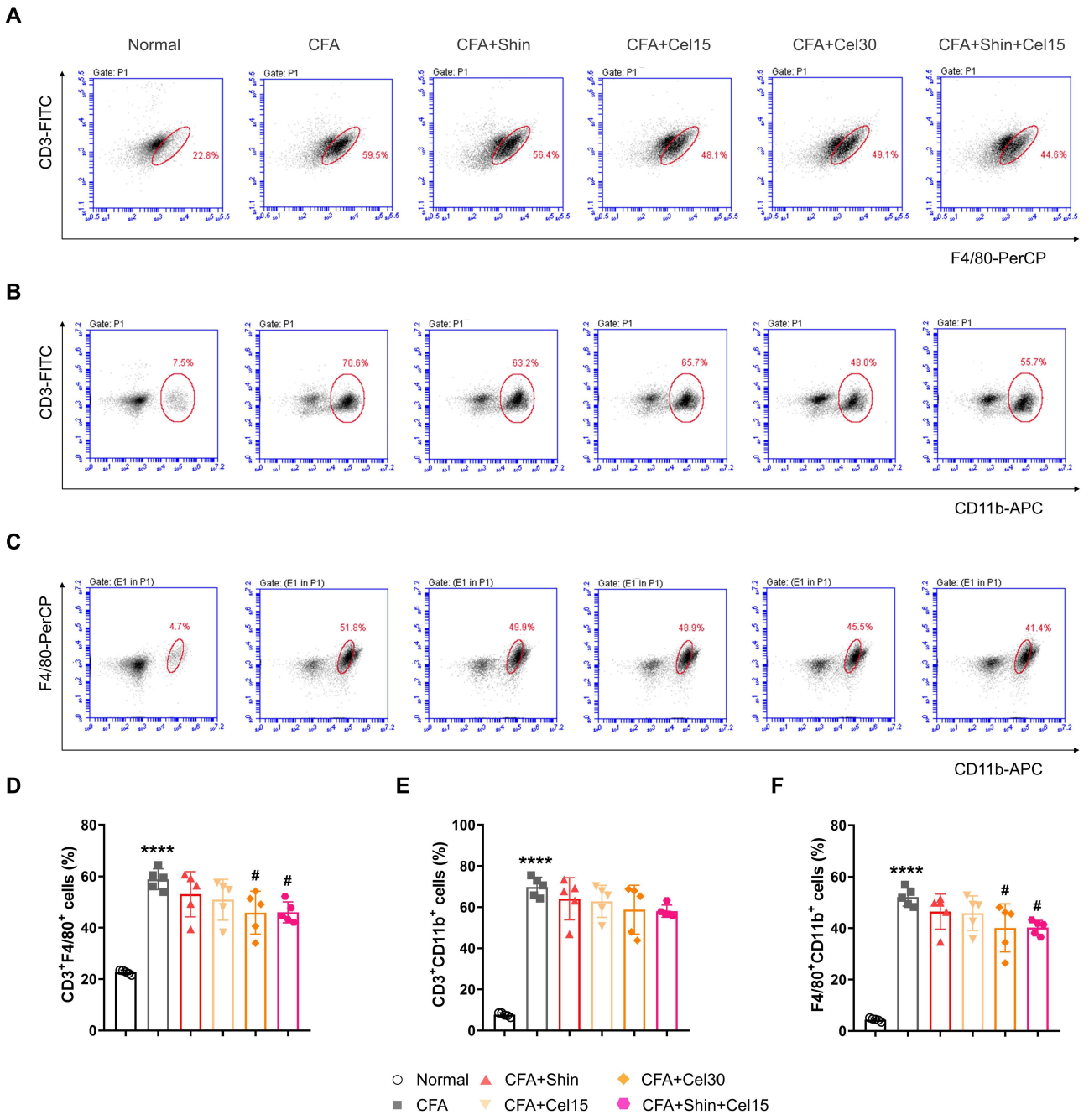

Figure 4 Effect of combined Shinbaro and celecoxib treatment on peripheral inflammation induced by CFA. Fluorescence-activated cell sorting (FACS) was performed 3 days after CFA or saline injection to evaluate immune cell infiltration in the hind paws of mice. Cells were stained with CD3, F4/80, and CD11b antibodies and then gated onto live cells as (**A** and **D**) $CD^+F4/80^+$ cells, (**B** and **E**) $CD3^+CD11b^+$ cells, and (**C** and **F**) $F4/80^+CD11b^+$ cells. n = 5/group. ****$p < 0.0001$ vs Normal, $^{\#}p < 0.05$ vs CFA. The results were analyzed using a one-way analysis of variance followed by Tukey's post-hoc test. Data are expressed as mean ± standard deviation.

Discussion

We investigated whether the combination of Shinbaro and celecoxib was more effective in suppressing pain and inflammation than either agent alone in a mouse model of CFA-induced inflammatory pain. We found that combination therapy effectively suppressed increases in pain sensitivity and hind paw thickness induced by CFA injection. Additionally, it attenuated the infiltration of monocytes and macrophages caused by CFA. Furthermore, the combination not only inhibited the activation of microglia and astrocytes, which contribute to neuroinflammation in the dorsal horn of the spinal cord, but also reduced the expression of pro-inflammatory cytokines and mediators. These results demonstrate

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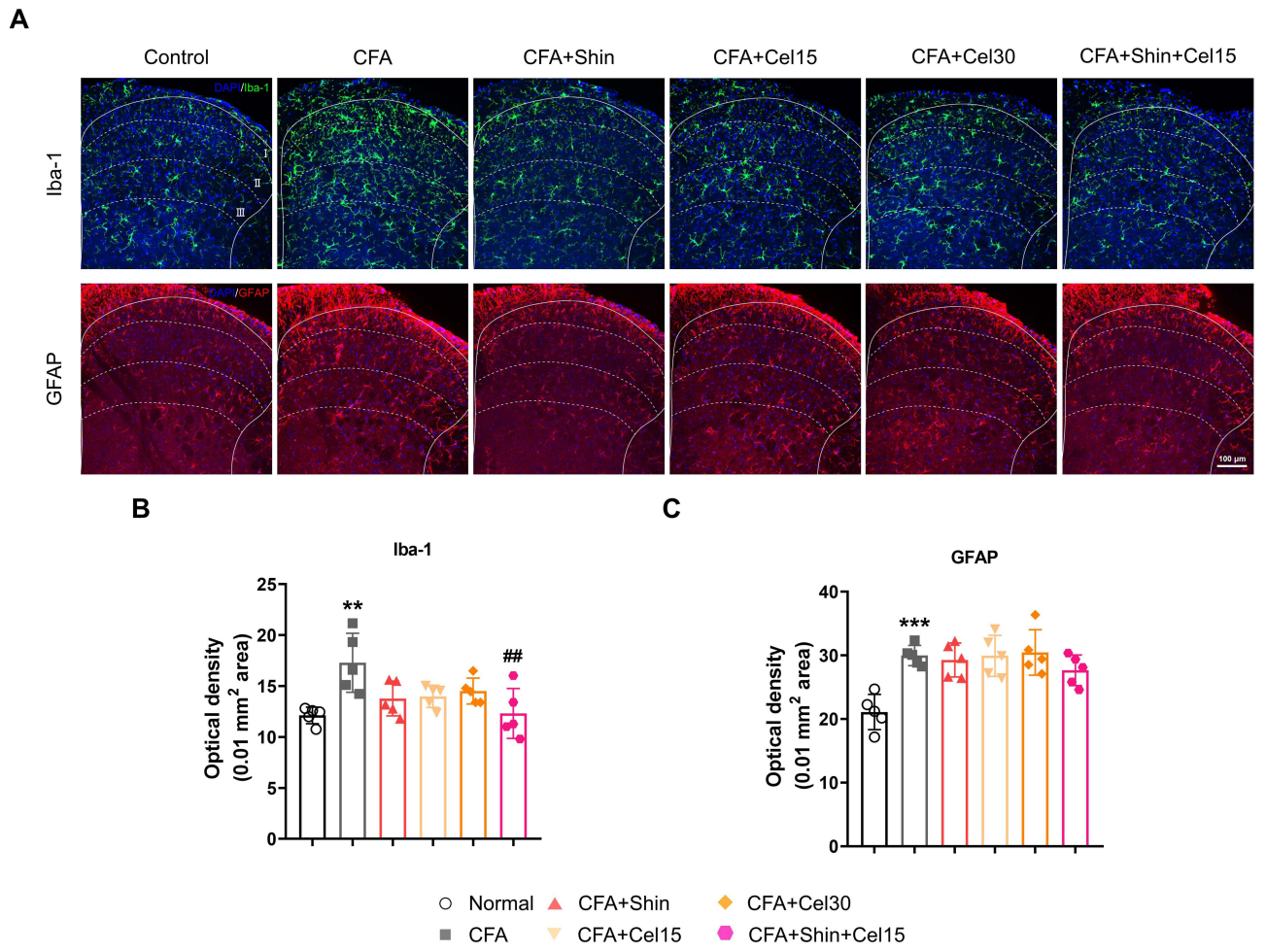

Figure 5 Effect of combined Shinbaro and celecoxib treatment on the activation of spinal microglia and astrocytes induced by CFA.Immunofluorescence staining was conducted on spinal cords extracted 3 days after CFA or saline injection. (**A**) The L4–L6 spinal cord was stained with Iba-1 and GFAP antibodies to observe microglia and astrocytes. Optical densities of (**B**) Iba-1 and (**C**) GFAP were measured on a grid (100 m × 100 m) of laminae I and II. Scale bar: 100 μm. n = 5/group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs Normal, $^{\#\#}p < 0.01$ vs CFA. All results were analyzed using a one-way analysis of variance followed by Tukey's post-hoc test. All data are presented as mean ± standard deviation.

that the combination of Shinbaro and celecoxib may have synergistic effects on pain relief and local inflammation suppression and may also reduce neuroinflammation that reinforces pain signals.

Celecoxib is commonly used to treat inflammatory pain, such as rheumatoid arthritis and osteoarthritis.[5] Combination therapy may have synergistic effects on suppressing pain and inflammation and could potentially reduce the incidence of adverse effects, such as cardiovascular disease, by allowing for the use of lower doses of celecoxib.[9–11,29,30] Preclinical and clinical studies have revealed extensive pharmacodynamic and pharmacokinetic interactions between bioactive compounds and herbal medicines.[31] For example, extracts of *Scutellaria baicalensis* have shown high efficacy and reduced toxicity due to synergistic interactions with conventional drugs.[31–34] Shinbaro is an herbal medicine known for its low toxicity and anti-inflammatory and analgesic effects on inflammatory pain, including rheumatoid arthritis and osteoarthritis.[12,13] Therefore, we investigated whether combined treatment with Shinbaro and celecoxib has a synergistic effect in suppressing pain and inflammation in a mouse model of CFA-induced inflammatory pain and explored the mechanism underlying this effect.

We used an inflammatory pain model to investigate the anti-inflammatory and analgesic effects of the combined Shinbaro and celecoxib treatment. Injection of CFA into the hind paws of mice induces mechanical hyperalgesia and paw edema due to increased peripheral inflammation.[35] In this study, the combination of Shinbaro and celecoxib not only reduced mechanical hyperalgesia but also decreased paw edema compared to either preparation alone.

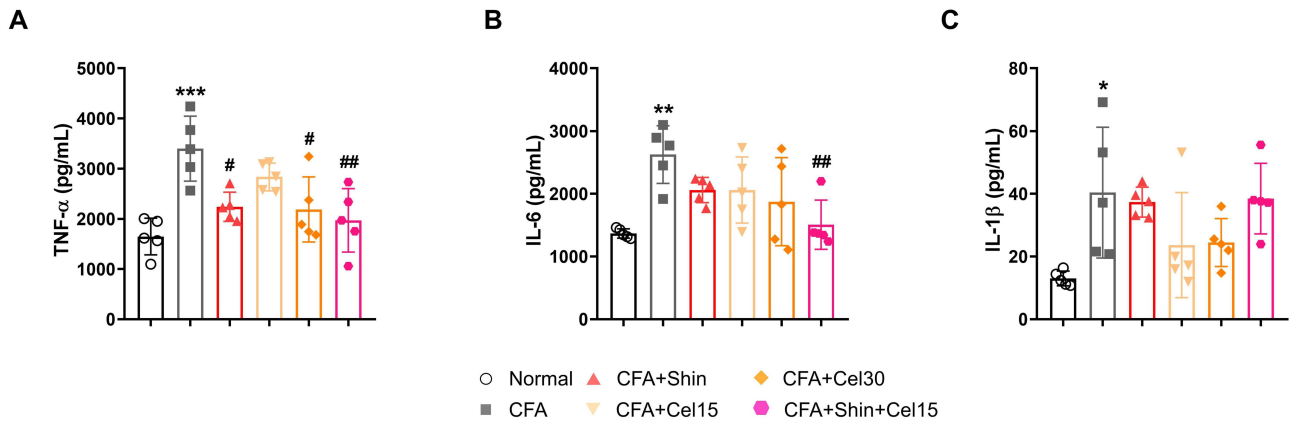

Figure 6 Effect of combined Shinbaro and celecoxib treatment on the release of pro-inflammatory cytokines induced by CFA.To measure the levels of pro-inflammatory cytokines in the L4–L6 spinal cord extracted 3 days after CFA or saline injection, (**A**) TNF-α, (**B**) IL-6, and (**C**) IL-1β expression was evaluated by using an enzyme-linked immunosorbent assay kit, respectively. n = 5/group. *$p < 0.01$, **$p < 0.01$, ***$p < 0.001$ vs Normal, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs CFA. These data were analyzed using a one-way analysis of variance followed by Tukey's post-hoc test. All graphs are presented as mean ± standard deviation.

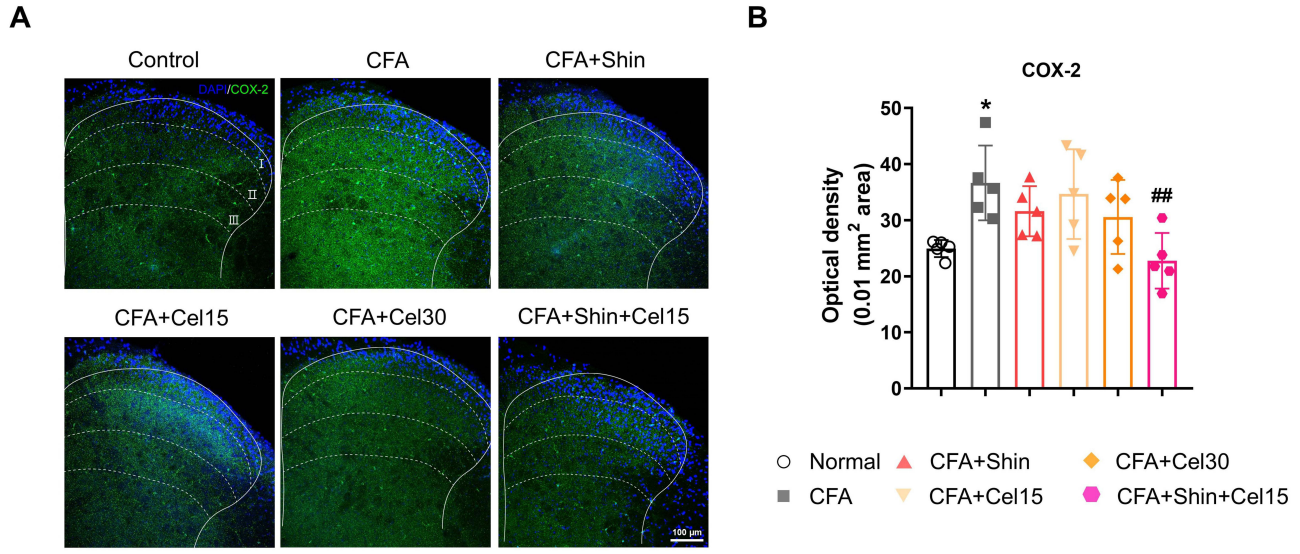

Figure 7 Effect of combined Shinbaro and celecoxib treatment on the increased expression of CFA-induced pro-inflammatory mediators. (**A**) The expression of COX-2 was observed in the L4–L6 spinal cord using immunofluorescence staining. (**B**) The optical density of COX-2 was evaluated on a grid (100 × 100 μm) in laminae I and II. Scale bar: 100 μm. n = 5/group. *$p < 0.05$ vs Normal, $^{\#\#}p < 0.01$ vs CFA. This result was a one-way analysis of variance followed by Tukey's post-hoc test. Data are expressed as mean ± standard deviation.

Additionally, the combination therapy exhibited a synergistic effect on the improvement rates of both mechanical hyperalgesia and paw edema.

We also analyzed the infiltration of monocytes and macrophages in the right hind paw using flow cytometry, which showed that infiltration into tissues peaked 2–3 days after CFA injection and was subsequently attenuated by combination therapy.[36] Macrophages serve as a bridge between the innate and adaptive immune systems and play a pivotal role in innate immunity.[37] They release inflammatory cytokines and chemokines, and their receptors are thought to interact bidirectionally with macrophage-nociceptive neurons, macrophage-microglia, and microglia-nociceptive neurons. The result of these interactions is macrophage homing, continuous activation of nociceptive transmission neurons, macrophage migration, and microglial activation, and thus, peripheral macrophages transmigrate into the spinal cord,[38–40] where they may induce neuroinflammation and contribute to the development and maintenance of pain sensitization.[41]

To investigate the effect of the combination of Shinbaro and celecoxib on neuroinflammation, we examined the activation of microglia and astrocytes in the dorsal horn of the spinal cord. Glial cells, such as microglia and astrocytes,

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are the most abundant cells in the central nervous system (CNS) and regulate neuronal function and signaling. Additionally, glial cells may be involved in neuroinflammation and contribute to the development of pain.[41,42] Microglia, which are macrophage-like cells in the CNS, are associated with various inflammatory and neurological disorders. Their activation increases pain sensitivity by producing pro-inflammatory cytokines and impairing the balance of excitatory and inhibitory synaptic transmission.[43–45] In this study, combination therapy inhibited the activation of microglia induced by CFA injection in laminae I–II of the dorsal horn. Upon the onset of painful peripheral stimulation, nociceptive neurons are activated, and nociceptive signals are transmitted via axons of primary afferent nerve fibers (C- and Aδ-fibers) to nerve terminals in laminae I–II of the dorsal horn.[46–48] Damage to primary afferent nerve fibers following peripheral injury activates microglia, which are primarily distributed in laminae I–III of the spinal cord, ultimately enhancing the excitability of spinal pain sensory circuits.[49–52]

Given that microglial activation releases pro-inflammatory cytokines, we observed the expression of TNF-α, IL-6, and IL-1β in the spinal cord. The combination of Shinbaro and celecoxib reduced the expression of TNF-α and IL-6 in the spinal cord to a greater extent than either treatment alone. Increased TNF-α expression in the spinal cord contributes to the development of mechanical allodynia and thermal hypersensitivity by enhancing excitatory neurotransmission in lamina II neurons in the dorsal horn of the spinal cord in a TNF receptor 1-dependent manner.[53,54] Additionally, intrathecal injection of IL-6 induces hyperalgesia and allodynia by acting on IL-6 receptors in spinal neurons.[55,56] Furthermore, we found that combination therapy reduced COX-2 expression in the spinal cord more than either treatment alone. Therefore, these results suggest that the combination of Shinbaro and celecoxib can induce pain relief by downregulating the release of pro-inflammatory molecules through the inhibition of spinal microglial activity.

Although the results suggest that the combination of Shinbaro and celecoxib has a synergistic effect in reducing pain and inflammation in a CFA-induced inflammatory pain mouse model, this study had some limitations. Specifically, the mechanisms underlying the synergistic effect of the combination therapy were not elucidated. For example, the impact of Shinbaro on the pharmacokinetic profile of celecoxib, such as absorption, distribution, metabolism, and elimination, was not investigated. Although celecoxib is a selective COX-2 inhibitor, the mechanism of action of Shinbaro remains unknown, and the pharmacological action of combination therapy warrants further investigation. Additional studies are required to address these limitations.

Conclusions

In this study, we demonstrated the anti-inflammatory and analgesic effects of a combination of Shinbaro and celecoxib on inflammatory pain and explored the underlying molecular mechanisms. This combination therapy may offer a viable treatment option for patients with inflammatory pain due to its potential to reduce side effects and expand the range of available treatment options.

Abbreviations

NSAIDs, nonsteroidal anti-inflammatory drugs; COX-2, cyclooxygenase-2; IL, interleukin; CFA, complete Freund's adjuvant; CMC, carboxymethyl cellulose; PBS, phosphate-buffered saline; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adaptor molecule 1; ANOVA, analysis of variance; CNS, central nervous system.

Data Sharing Statement

All datasets generated for this study have been included in the manuscript and Supplementary materials.

Ethics Approval

All experimental procedures were conducted in accordance with institutional guidelines and regulations for the care and use of laboratory animals, and all experimental processes were approved by the Jaseng Animal Care and Use Committee [IACUC No. JSR-2024-01-003-A].

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Disclosure

The authors declare that they have no competing interests in this work.

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