

Efficacy of a standardized *Centella asiatica* (ECa 233) extract against allodynia in a mouse model of temporomandibular osteoarthritis

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ABSTRACT: Prolonged inflammation causing tissue injury can induce chronic pain hypersensitivity (allodynia and hyperalgesia). Osteoarthritis of the temporomandibular joint (TMJ-OA) is a common cause of chronic allodynia encountered in dental practice, but many currently available treatments induce intolerable side effects. In this study, we investigated the potential efficacy of a standardized *Centella asiatica* extract (ECa 233) on allodynia in a TMJ-OA mouse model established by an injection of complete Freund's adjuvant (CFA) into the TMJ. After CFA injection, animals daily received oral administration of vehicle, 0.14 g/kg ibuprofen (positive control), 30 mg/kg ECa 233, or 100 mg/kg ECa 233. Behavioral pain responses were examined by air-puff tests before and after CFA injection on days 3, 7, 14, 21, and 28. On day 28, TMJ-OA pathology was assessed by changes in articular cartilage thickness and graded according to the Osteoarthritis Cartilage Histopathology Assessment (OCHA) system. In the CFA + vehicle group, pain response scores increased gradually, reaching statistical significance compared to untreated Sham controls on days 14, 21, and 28. On day 28, OCHA grade score was 3.5 ± 0.35 , and articular cartilage thickness was reduced compared to the Sham group. Both ECa 233 doses significantly attenuated pain response scores and also slowed degeneration of the TMJ with efficacy comparable to ibuprofen. We conclude that ECa 233 can protect against mechanical allodynia and cartilage degeneration in a mouse model of TMJ-OA.

KEYWORDS: temporomandibular joint osteoarthritis, chronic pain, complete Freund's adjuvant, *Centella asiatica*

INTRODUCTION

Temporomandibular joint osteoarthritis (TMJ-OA) is a progressive degenerative joint disease characterized by inflammation, destruction of articular cartilage, afferent nerve sensitization, and ensuing chronic inflammatory pain [1]. It is the most common cause of chronic TMJ pain and can occur at any age but is especially frequent in dental patients older than 40 years [2, 3]. The primary symptom is pain at the TMJ, and surrounding areas evoked by normally innocuous stimuli such as air flow, defined as mechanical allodynia [4, 5]. Nonsteroidal anti-inflammatory drugs (NSAIDs) can effectively relieve the pain and minimize inflammation associated with TMJ-OA, but long-term use may have adverse effects including peptic ulcer and increased risks for kidney and cardiovascular diseases [6]. Further, a substantial number of cases are refractory to NSAIDs, necessitating the introduction of more effective and safer alternatives.

Among potential treatments with well documented efficacy and safety for other conditions are traditional herbal extracts containing concentrates of bioactive phytochemicals. *Centella asiatica* (L.) Ur-

ban (Indian pennywort, Gotu kola or Bua-bok) is a common component of traditional herbal medicines with reported anti-inflammatory and antinociceptive properties [7]. The major bioactive components of *Centella asiatica* extracts are triterpenoid glycosides including asiaticoside and madecassoside [7]. However, the analgesic efficacy of these preparations is inconsistent [7] likely due to the variation in active ingredient concentrations. To overcome this problem, guidelines have been established for controlling the preparation process, yielding a standardized *Centella asiatica* extract termed ECa 233 containing not less than 80% w/w triterpenoid glycosides and a madecassoside to asiaticoside ratio of $1.5 \pm 0.5:1$ [8].

This ECa 233 preparation has demonstrated a favorable safety profile in both acute and chronic toxicity tests. Mice and rats receiving 10–1000 mg/kg for 7 days or 90 days by oral administration showed no alterations in hepatocyte histopathology [9]. Further, ECa 233 neither activated nor inhibited hepatic enzymes that can eliminate or detoxify other major analgesic such as paracetamol and NSAIDs [8], suggesting no interference with drug metabolism. Moreover, ECa 233 at 30 and 100 mg/kg has been shown to reduce

experimental thermal pain responses in rodents as assessed by the tail flick test [10], while an oral paste containing ECa 233 significantly decreased inflammation and pain in patients with aphthous ulcers [11]. Thus, ECa 233 appears to be a reliable antinociceptive and anti-inflammatory treatment. A few studies have also investigated the antinociceptive efficacy of *Centella asiatica* extract on knee joint osteoarthritis [12], but ECa 233 has not been examined for effects on pain associated with TMJ-OA.

Osteoarthritis results from the local release of inflammatory factors such as bradykinin, histamine, serotonin, and prostaglandins in response to minor tissue damage, leading to more extensive tissue injury and a self-perpetuating cycle of progressive tissue degeneration and increasing pain severity [13]. We therefore speculated that a TMJ-OA model could be established by local exposure to an exogenous pro-inflammatory agent such as complete Freund's adjuvant (CFA). In fact, previous studies have demonstrated that CFA injection into the TMJ of mice causes inflammation and induces behavioral signs of chronic TMJ-OA-like pain in response to non-noxious stimuli [14, 15] possibly by inducing sustained activation of primary pain afferents. Chronic activation of these afferents can result in peripheral sensitization and ultimately in central pain sensitization (allodynia and hyperalgesia) [16].

The aim of the present study is to examine the potential efficacy of ECa 233 for reducing behavioral pain responses in a TMJ-OA mouse model established by daily unilateral CFA injection. Moreover, we also examined the structural changes of the TMJ induced by CFA injection to confirm the validity of the model. To our best knowledge, this study is the first to demonstrate the possible benefits of *Centella asiatica* extract ECa 233 on both TMJ-OA pathogenesis and allodynia.

MATERIALS AND METHODS

Study groups and experimental design

This study was approved by the Animal Care and Use Committee, Faculty of Medicine, Siriraj Hospital, Mahidol University (SI-ACUP014/2561) and was performed according to the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) Guidelines. The sample size was calculated to provide 80% power ($1-\beta$) with a 95% confidence interval ($\alpha = 0.05$) [14]. Thirty adult male ICR mice (6 weeks old, 28–32 g initial body weight) were randomly divided into 5 groups of 6: a Sham treatment group and 4 CFA groups also receiving vehicle, ibuprofen, or one of 2 ECa 233 doses (30 and 100 mg/kg). Each group was housed in separate cages under controlled temperature ($22 \pm 2^\circ\text{C}$), humidity ($45\% \pm 15\%$), and light/dark cycle (12 h/12 h). The experimental design is illustrated in Fig. 1.

Experimental design

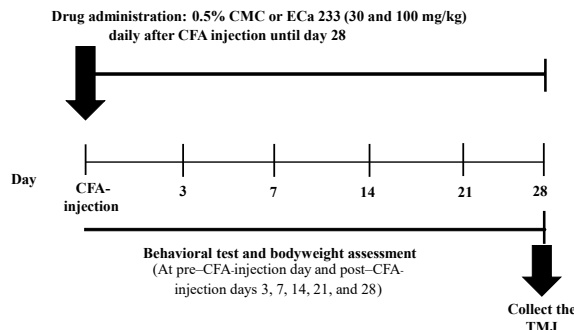


Fig. 1 Experimental design. CFA, complete Freund's adjuvant; CMC, carboxymethyl cellulose; and TMJ, temporomandibular joint (30 and 100 mg/kg) daily after CFA injection until day 28.

Development of the TMJ-OA mouse model

To induce a condition resembling TMJ-OA, mice were first injected intraperitoneally with sodium pentobarbital (60 mg/kg) for anesthesia and then with 10 μl of pure normal saline (NSS) (Sham group) or NSS containing 50% CFA (1 mg/ml) (F5881; Sigma-Aldrich, St. Louis, MO, USA) into the right TMJ. Injections were conducted once at the beginning of the experiment according to the previous studies [14, 17, 18]. The anatomical landmark for injection was also described in a previous study [17]. Briefly, to identify the TMJ by palpation, the local hairs were trimmed with a pair of scissors, and a 30-gauge needle was inserted through the facial skin until the tip reached the zygomatic arch. The needle was then moved slowly until it passed under the edge of the arch and entered the TMJ space. After the needle reached the TMJ space, 10 μl of NSS with or without 50% CFA was injected slowly over a period of 5 s under control of a Hamilton syringe [14, 17]. The body weight of each mouse was monitored throughout the 28-day experimental period to determine whether TMJ pain alters eating habits or general health.

Drug administration and ECa 233 preparation

After the CFA injection at the beginning, mice in the Sham and CFA + vehicle groups daily received 0.3 ml of 0.5% carboxymethyl cellulose (CMC) by oral administration. The other 3 CFA groups also daily received an equal volume of CMC containing either (i) 0.14 g/kg ibuprofen, (ii) 30 mg/kg ECa 233, or (iii) 100 mg/kg ECa 233. The ECa 233 was obtained from Siam Herbal Innovation (Lot No. MRA0511401; Bangkok, Thailand), and ECa 233 suspensions in CMC were prepared daily. All injections and oral administrations

were conducted at around 8 am.

Behavioral tests for pain sensitivity

Pain sensitivity was evaluated by applying a 10-psi air puff controlled by a pneumatic pump. Animals were treated in a box container as described in the previous studies [14, 15, 19]. The 10-psi air puff was chosen because it was shown to elicit substantial mechanical allodynia in CFA-injected TMJ-OA mice [15], while a stronger air puff (> 10 psi) was inherently noxious and so by definition cannot measure allodynia [14, 15, 19]. Tests were administered both before and after CFA injections on days 3, 7, 14, 21, and 28. The air puff was delivered to the right whisker pad (ipsilateral to CFA injection) as described in a previous study [14]. The 28-day measurement period was chosen to assess pain hypersensitivity during both the acute and chronic phases (more than 14 days in animal models according to [20]). We tested 12 times and then recorded response scores as follows: no behavioral response = 0, head withdrawal = 0.25, face grooming once = 1, and face grooming of > 3 times = 1.5 [14, 19]. Response scores were added to obtain the total ipsilateral response scores (maximum of 18).

Structural changes of the TMJ

After day 28, all mice were deeply anesthetized and transcardially perfused with 250 ml ice-cold phosphate-buffered saline (PBS; 0.1 M, pH 7.4). The head was removed and immersed in 4% paraformaldehyde in PBS (0.1 M, pH 7.4), followed by removal of the skin and opening of the cervical bone. The right TMJ was then excised and decalcified in 10% formic acid for 7 days before paraffin embedding, sectioning, and histological staining. The TMJ structures including the condylar head, articular disk, and temporal bone were sagittally sectioned at 10- μ m thickness using a microtome (Global Medical Instrumentation Inc., Ramsey, MN, USA). Sections were deparaffinized, stained with hematoxylin and eosin (HE, SC-396330; Santa Cruz Biotech, Dallas, TX, USA) diluted 1:100 in PBS, and examined under light microscopy (Carl Zeiss Microimaging GmbH, AxioVision 40 version 4.8.2.0, Germany). Sections were collected at intervals of approximately 10 (10th, 20th, 30th, 40th, 50th, and 60th sections) to measure the articular cartilage thickness and grade the degree of TMJ injury by the Osteoarthritis Cartilage Histopathology Assessment (OCHA) system [21, 22]. Grading was conducted independently by 3 observers using the following scoring criteria [14, 23]: surface intact/cartilage morphology intact = 0, surface intact with abrasion on the superficial layer = 1, surface discontinuity = 2, vertical fissures or clefts = 3, surface erosion = 4, sclerotic bone within a denuded surface = 5, and gross deformation = 6. We measured the articular cartilage thickness in the same slides (10th, 20th, 30th, 40th, 50th, and 60th)

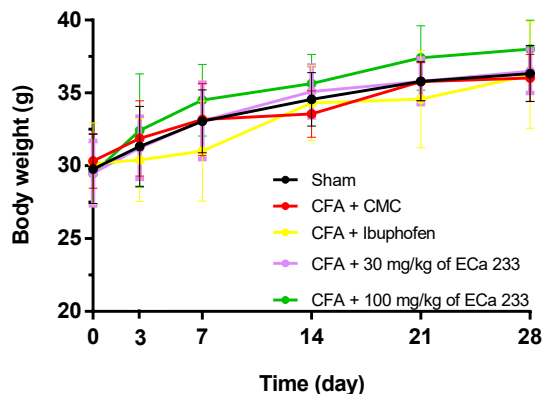


Fig. 2 Effect of CFA-induced temporomandibular joint osteoarthritis (TMJ-OA) on body weight at any time points during the injection protocol. Data are expressed as mean \pm SEM, $n = 6$ per group.

examined by the OCHA system. The articular cartilage thickness was measured independently by 3 observers blinded to group allocation at positions where the mandibular condyle head was 20%, 50%, and 80% of total horizontal thickness using ImageJ (<https://imagej.nih.gov>) [24].

Statistical analysis

All data, represented from triplicate experiments, are presented as mean \pm standard error of the mean (SEM). Datasets were first tested for normality using the Kolmogorov-Smirnov test. Group means were then compared by two-way analysis of variance (ANOVA) with post hoc Fisher's least significant difference tests for pair-wise comparisons. A $p < 0.05$ (two-tailed) was considered statistically significant for all tests. All statistical calculations were conducted using SPSS version 26.0 (IBM, Chicago, IL, USA).

RESULTS

Body appearance: Weight

There were no significant differences in body weight among Sham, CFA + vehicle, CFA + ibuprofen, CFA + 30 mg/kg ECa 233, and CFA + 100 mg/kg ECa 233 groups after 28 days of treatment (Fig. 2 and Table 1), indicating that induced pain (below) did not interfere with animal feeding or otherwise impair general health and development.

ECa 233 attenuates mechanical allodynia development in a chronic TMJ-OA model

Fig. 3 illustrates the time course of behavioral pain responses to the 10-psi air-puff test for mechanical allodynia. As expected, the Sham group showed the lowest pain response scores, and responses remained at baseline throughout the 28-day period. In contrast, the CFA + vehicle group demonstrated a progressive

Table 1 Changes in weekly body weight (g) among treatment groups.

Group	Pre-injected	Day 3	Day 7	Day 14	Day 21	Day 28
Sham	27.78 ± 2.38	31.33 ± 2.73	33.06 ± 2.16	34.56 ± 1.84	35.80 ± 1.34	36.33 ± 1.92
CFA + CMC	30.33 ± 1.87	31.87 ± 2.58	33.17 ± 2.47	33.56 ± 1.59	35.78 ± 1.31	36.03 ± 1.62
CFA + ibuprofen	30.16 ± 2.77	30.40 ± 2.84	31.01 ± 3.43	34.31 ± 2.56	34.58 ± 3.34	36.22 ± 3.85
CFA + 30 mg/kg ECa 233	29.49 ± 2.21	31.23 ± 2.14	33.08 ± 2.62	35.10 ± 1.79	35.80 ± 1.57	36.49 ± 1.50
CFA + 100 mg/kg ECa 233	29.47 ± 2.12	32.42 ± 3.89	34.50 ± 2.46	35.64 ± 2.01	37.40 ± 2.21	38.44 ± 2.18

Values presented are mean ± SEM ($n = 6$).

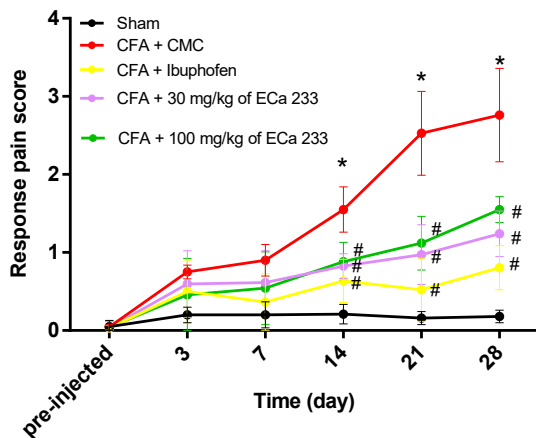


Fig. 3 Effect of ECa 233 on pain response scores at the different time points after CFA-induced temporomandibular joint osteoarthritis (TMJ-OA). Data are expressed as mean ± SEM, $n = 6$ per group. * $p < 0.05$ compared to the Sham group. # $p < 0.05$ compared to the CFA + CMC group.

increase in pain response that was significantly higher than in Sham mice on injection days 14 [$F(4, 45) = 13.823$, $p < 0.05$], 21 [$F(4, 45) = 15.213$, $p < 0.05$], and 28 [$F(4, 45) = 19.149$, $p < 0.05$]. Daily ibuprofen, 30 mg/kg ECa 233, and 100 mg/kg ECa 233 co-treatments all significantly reduced pain responses to air puffs on days 14, 21, and 28 compared to CFA + vehicle group (CFA + Ibuprofen: [$F(4, 45) = 15.139$, $p < 0.05$], [$F(4, 45) = 16.134$, $p < 0.05$], and [$F(4, 45) = 17.901$, $p < 0.05$]; CFA + 30 mg/kg of ECa 233: [$F(4, 45) = 15.215$, $p < 0.05$], [$F(4, 45) = 15.652$, $p < 0.05$], and [$F(4, 45) = 14.592$, $p < 0.05$]; CFA + 100 mg/kg of ECa 233: [$F(4, 45) = 18.252$, $p < 0.05$], [$F(4, 45) = 16.358$, $p < 0.05$], and [$F(4, 45) = 16.014$, $p < 0.05$], respectively), indicating significant reduction of mechanical allodynia. Further, there were no significant differences in pain response among ibuprofen and ECa 233 groups on any day, suggesting that ECa 233 was roughly as effective as the ubiquitous clinical analgesic.

ECa 233 also alleviates CFA-induced TMJ injuries

Injuries to the TMJ induced by CFA injection were assessed on histological sections according to the OCHA

system. Fig. 4 shows the structural changes of the TMJ condylar head after all treatment protocols. After the CFA injection, the surface of the condylar head had eroded and detached. Mean OCHA system grading score for the right TMJ was 3.5 ± 0.35 after 28 days in the CFA + vehicle group but was 0 in the Sham group, ibuprofen group, and both ECa 233 (30 and 100 mg/kg) groups. Thus, ECa 233 also prevented TMJ condyle head degeneration as effectively as ibuprofen.

Further, CFA reduced while ECa 233 co-treatment partially preserved normal articular cartilage thickness at positions where the mandibular condyle head is 20%, 50%, and 80% of maximum horizontal thickness. The CFA + vehicle group exhibited a significantly reduced mean articular cartilage thickness on day 28 [$F(4, 45) = 12.823$, $p < 0.05$] compared to the Sham group, while articular cartilage thicknesses values did not differ among Sham, ibuprofen, and ECa 233 treatment groups (CFA + Ibuprofen: [$F(4, 45) = 10.339$, $p = 0.253$], CFA + 30 mg/kg of ECa 233: [$F(4, 45) = 11.034$, $p = 0.279$], and CFA + 100 mg/kg of ECa 233: [$F(4, 45) = 11.001$, $p = 0.265$]) (Fig. 5).

DISCUSSION

This is the first *in vivo* study demonstrating that a standardized extract of *Centella asiatica* (ECa 233) can attenuate pain hypersensitization (mechanical allodynia) and joint degeneration in a CFA injection model of TMJ-OA. During the CFA injection period, food and water intake were not impaired as evidenced by the normal increase in body weight. Stimulation with a normally innocuous air puff induced behavioral signs of pain on days 14, 21, and 28 of CFA + vehicle injection in accord with the previous studies showing that CFA can induce the excessive inflammation and pain hypersensitivity characteristic of TMJ-OA [19, 20]. Further, we provide additional validation of the model by showing that CFA caused degeneration of the TMJ articular cartilage surface, consistent with previous reports that articular cartilage thickness and mandible bone density were reduced after 21 and 28 days of CFA injection in mice [14, 25].

The pathophysiology of chronic TMJ-OA is still not completely clear but likely involves local release of pro-inflammatory factors such as bradykinin, histamine, serotonin, and prostaglandins [26]. Similarly, CFA

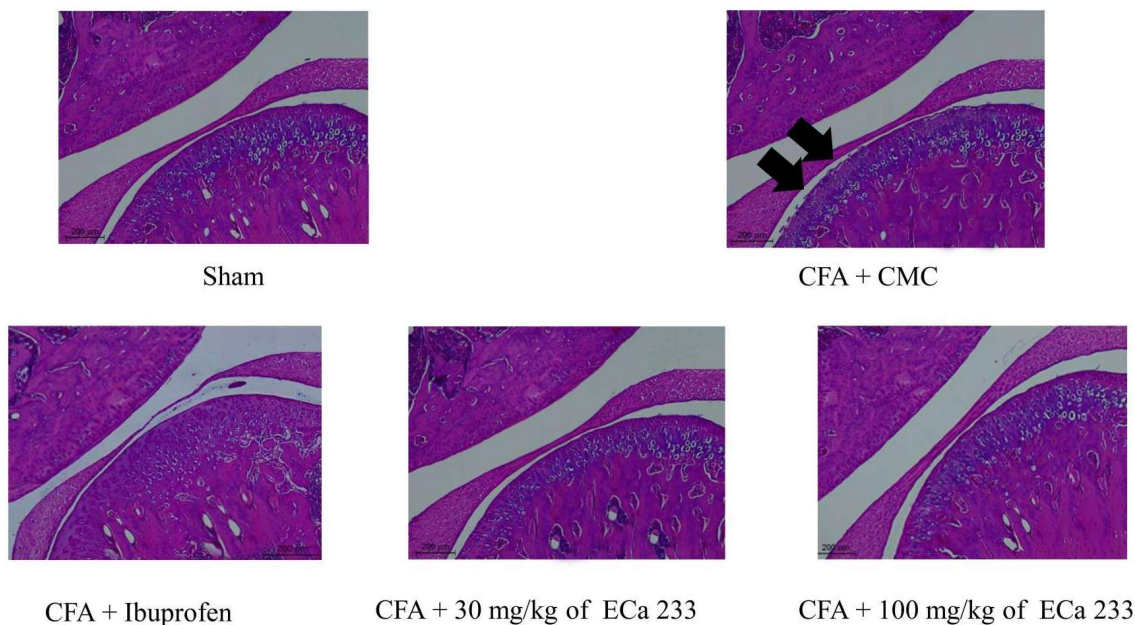
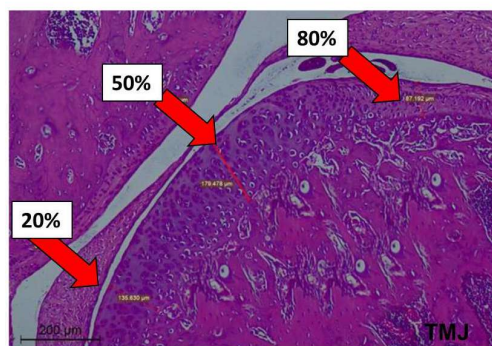


Fig. 4 Effect of ECa 233 on degeneration of the ipsilateral TMJ condyle head articular surface. Representative images of hematoxylin and eosin (HE)-stained sections of the TMJ after 28 days of the CFA injection plus vehicle, ibuprofen, or ECa 233 (scale bar = 200 μ m). The black arrows indicate the degeneration of the ipsilateral articular surface.

(a)



(b)

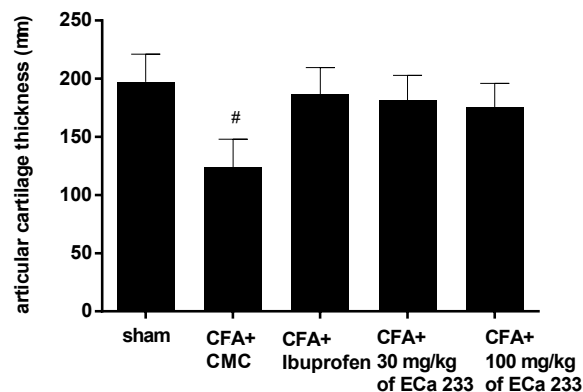


Fig. 5 Effect of ECa 233 on articular cartilage thickness of the condyle head TMJ. (a) Representative measurement of articular cartilage thickness at positions corresponding to 20%, 50%, and 80% of maximum mandible condyle head thickness (red arrows) from hematoxylin and eosin (HE)-stained sections prepared on day 28 of the experimental protocol (scale bar = 200 μ m), (b) mean values of articular cartilage thickness in the different groups. Data are expressed as mean \pm SEM, $n = 6$ per group. [#] $p < 0.05$ compared to the Sham group.

injection induces the release of inflammatory mediators that chronically activate primary nerve afferents. Accumulation of these factors and ensuing tissue damage ultimately reduces nerve firing threshold and promotes ectopic synapse formation (plasticity), resulting in increased pain sensitivity at the peripheral level (peripheral sensitization) and eventually at the spinal level (central sensitization) [27]. In allodynia, air flow or light touch can evoke pain sensation possibly by activating low-threshold myelinated A fibers mediating

touch sensation that spouts axons and synapse on nociception-transmitting interneurons in laminae I and II of the trigeminal subnucleus caudalis (TNC, which also receives projections from nociceptive A δ and C fibers) [28]. Further study is required to determine if CFA injection induces and ECa 233 reverses myelinated A fiber innervation of the TNC.

The majority of TMJ-OA patients eventually develop abnormalities in TMJ bone structure [26, 27]. These changes may result from chronic inflammation

as experimentally evoked inflammation in the TMJ resulted in degeneration of the condylar head as well as pain hypersensitivity [20, 29, 30]. Based on these findings, we speculated that CFA would alter TMJ structure via uncontrolled inflammation, thereby mimicking the pathogenesis and clinical features of advanced TMJ-OA [20, 29]. Our previous study demonstrated that unilateral CFA injection can induce chronic inflammation in the ipsilateral joint, and allodynia was observed primarily on the ipsilateral side in the current study. Furthermore, this mechanical allodynia was sustained for longer than 14 days, defined as chronic for animal models. We suggest that these long-lasting changes represent central sensitization as Rotpenpian and colleagues reported significantly enhanced phosphorylated cAMP response element-binding protein (p-CREB) and microglial activation in the TNC on day 28 of CFA injection [14], indicating long-term hyperactivation of nociceptive pathways.

Both the clinical analgesic ibuprofen and ECa 233 (30 and 100 mg/kg) significantly reduced pain response scores compared to vehicle. Moreover, both treatments also protected the TMJ from degeneration as indicated by OCHA system grading scores and articular cartilage thickness measurements of TMJ sections, which are the most reliable assessments of osteoarthritis in animal models [5, 31]. Ibuprofen is a drug of choice for pain relief and can protect against osteoarthritis development by blocking COX enzymes and ensuing generation of inflammatory mediators [32–34]. Similarly, the bioactive ingredients in *Centella asiatica* extracts have demonstrated both anti-inflammatory and antinociceptive activities in preclinical studies. For example, like ibuprofen treatment, oral treatment with 100 mg/kg *Centella asiatica* extract reduced subsequent carrageenan- and prostaglandin-induced hind foot edema and pain in rats [7]. *Centella asiatica* extract also significantly reduced expression levels of the pro-inflammatory cytokines' tumor necrosis factor- α and interleukin-6 in lipopolysaccharide-treated HEK-293 cells [35].

The active ingredients of ECa 233 (which contains $\geq 80\%$ w/w triterpenoid glycosides and a 1.5:1 ratio of madecassoside to asiaticoside) also possess anti-inflammatory and antinociceptive activities when used alone. For instance, oral madecassoside (40 mg/kg) administration reduced the clinical pain scores of mice with induced rheumatoid arthritis of the tail joint and also minimized COX-2 activity and production of prostaglandins, tumor necrosis factor- α , and interleukin-6 [35]. Similarly, oral treatment with 10 mg/kg asiaticoside reduced capsaicin-induced paw pain in mice [36] possibly by increasing release of gamma aminobutyric acid (GABA), the major inhibitory neurotransmitter in the CNS. Indeed, an aqueous extract of *Centella asiatica* elevated the activity of

the GABA synthesis enzyme, glutamic acid decarboxylase (GAD) [37]. Moreover, ECa 233 was reported to activate GABAergic synapses in the amygdala [38]. Increased GABA activity in the CNS may block mechanical allodynia by interfering with the sensitization processes at the neural level. *Centella asiatica* also reduced pain in an animal model of migraine by blocking the serotonin (5-HT) receptors, 5-HT_{1A} and 5-HT_{1B} [39], and inducing activation of ERK1/2 and Akt signaling pathways [40]. Additional studies are needed to identify the molecular pathways underlying experimental allodynia in this TMJ-OA model and the therapeutic mechanisms of ECa 233 components.

This study has several limitations. First, pain in experimental animals can only be measured by behavioral responses, and behavioral scoring can be highly subjective. Also, we did not examine the progression of articular cartilage damage during the experiment to assess associations with pain responses (on day 3, 7, 14, and 21) as our primary aim was to investigate behavioral and structural changes during the chronic phase (after 28 days of CFA injection). Moreover, we have not yet examined the potential molecular mechanisms underlying these effects of ECa 233 such as reduction of pro-inflammatory cytokine accumulation in the TMJ, suppression of microglial activation in the spinal cord, and downregulation of p-CREB in the TNC.

CONCLUSION

A standardized *Centella asiatica* extract (ECa 233) protects against pain hypersensitivity and TMJ cartilage degeneration in a mouse model of chronic TMJ-OA without apparent systemic toxicity. Therefore, clinical trials investigating its efficacy and safety for chronic pain management in OA and other disorders are warranted.

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