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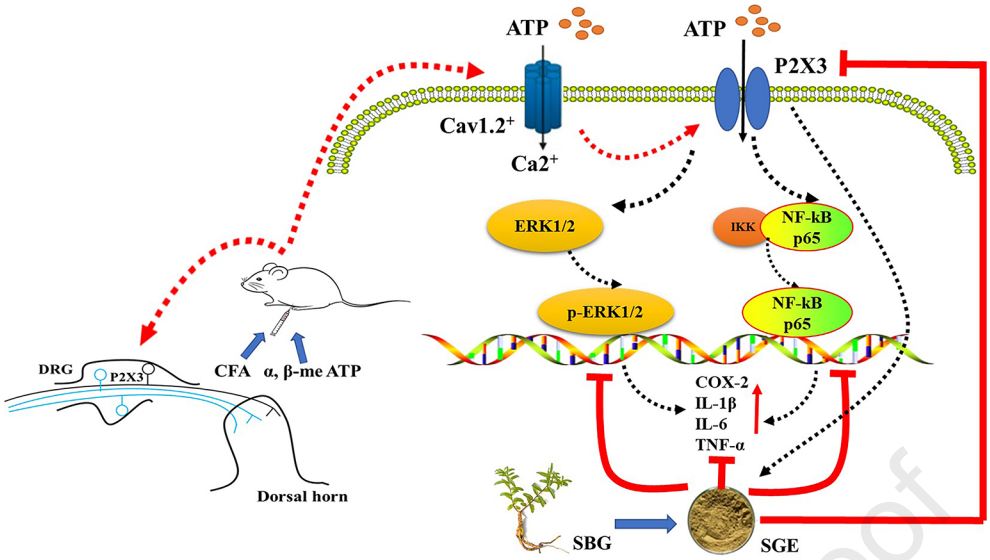
Credit author statement

LG and J-XZ provided the concept and designed the study. J-XZ performed the experiments and wrote the draft of the manuscript. X-MQ, J-Z and LG provided oversight. LG revised the manuscript and approved the manuscript submission. All authors read and approved the final manuscript. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.



The ethanol extract of *Scutellaria baicalensis* Georgi attenuates complete Freund's adjuvant (CFA)-induced inflammatory pain by suppression of P2X3 receptor

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Abstract:

Ethnopharmacological relevance: Scutellaria baicalensis Georgi (SBG) is a perennial herb with anti-inflammatory, antibacterial, and antioxidant activities, which is traditionally used to treat inflammation of respiratory tract and gastrointestinal tract, abdominal cramps, bacterial and viral infections. Clinically, it is often used to treat inflammatory-related diseases. Research has shown that the ethanol extract of *Scutellaria baicalensis* Georgi (SGE) has anti-inflammatory effect, and its main components baicalin and baicalein have analgesic effects. However, the mechanism of

SGE in relieving inflammatory pain has not been deeply studied.

Aim of the study: This study aimed to evaluate the analgesic effect of SGE on complete Freund's adjuvant (CFA)-induced inflammatory pain rats, and to investigate whether its effect on relieving inflammatory pain is associated with regulation of P2X3 receptor.

Materials and methods: The analgesic effects of SGE on CFA-induced inflammatory pain rats were evaluated by measuring mechanical pain threshold, thermal pain threshold, and motor coordination ability. The mechanisms of SGE in relieving inflammatory pain were explored by detecting inflammatory factors levels, NF-κB, COX-2 and P2X3 expression, and were further verified by addition of P2X3 receptor agonist (α, β me-ATP).

Results: Our results revealed that SGE can notably increase the mechanical pain threshold and thermal pain threshold of CFA-induced inflammatory pain rats, and markedly alleviate the pathological damage in DRG. SGE could suppress the release of inflammatory factors including IL-1β, IL-6, TNF-α and restrain the expression of NF-κB, COX-2 and P2X3. Moreover, α, β me-ATP further exacerbated the inflammatory pain of CFA-induced rats, while SGE could markedly raise the pain thresholds and relieve inflammatory pain. SGE could attenuate the pathological damage, inhibit P2X3 expression, inhibit the elevation of inflammatory factors caused by α, β me-ATP. SGE can also inhibit NF-κB and ERK1/2 activation caused by α, β me-ATP, and inhibit the mRNA expression of P2X3, COX-2, NF-κB, IL-1β, IL-6 and TNF-α in DRG of rats induced by CFA coupled with α, β me-ATP.

Conclusions: In summary, our research indicated that SGE could alleviate CFA-induced inflammatory pain by suppression of P2X3 receptor.

Keywords: SGE; Inflammatory pain; P2X3; NF-κB; ERK1/2

Abbreviations:

SBG, Scutellaria baicalensis Georgi

SGE, the ethanol extract of Scutellaria baicalensis Georgi

CFA, complete Freund's adjuvant

ATP, adenosine triphosphate

P2X3, purinergic receptor P2X ligand-gated ion channel 3

DRG, dorsal root ganglion

SNL, spinal nerve ligation

NF-κB, nuclear factor-kappaB

ERK1/2, extracellular signal-regulated kinases 1/2

MAPK, mitogen-activated protein kinase

IL-1β, interleukin-1β

IL-6, Interleukin-6

TNF-α, tumor necrosis factor-α

PWT, paw withdrawal threshold

TWL, thermal withdrawal latency

PWL, paw withdrawal latency

UPLC-MS, ultra performance liquid chromatography-mass spectrometry

1. Introduction

Inflammatory pain is a common chronic pain in clinics, which is a complex and multifactorial disease (Djouhri et al., 2015; Sunet al., 2021; Yuan et al., 2018). The release of pro-inflammatory mediators such as prostaglandins and cytokines can sensitize peripheral pain neurons, produce noxious stimuli and sustain inflammation (Ji et al., 2014). Since the pathological basis of inflammatory pain is unknown, it is imperative to study the mechanism of inflammatory pain and seek for effective drugs.

Extracellular adenosine triphosphate (ATP) is a ligand-gated ion channel and an indispensable substance involved in the physiological and pathological functions of

pain (Burnstock et al., 2013; Samways et al., 2014). Patients with endometriosis and interstitial cystitis pain are accompanied by ATP release (Ding et al., 2017; Sun et al., 2006). When the organism is injured, ATP is released to activate P2X3 (purinergic receptor P2X ligand-gated ion channel 3) receptor, and the increase of Ca^{2+} concentration causes pain sensitivity (Nazıroğlu et al., 2020; Wang et al., 2021). P2X3 is highly expressed in sensory neurons of human dorsal root ganglion (DRG) (Xia et al., 2021; Yiangou et al., 2000). While P2X3 is also related to many types of pain such as diabetic pain, complete Freund's adjuvant (CFA)-induced inflammatory pain and spinal nerve ligation (SNL)-induced neuropathic pain (Guo et al., 2021; Xiang et al., 2019; Xu, et al., 2011). The activation of P2X3 receptor can induce inflammatory hyperalgesia in peripheral tissues (Oliveira et al., 2009; Prado et al., 2013; Schiavuzzo et al., 2015). Therefore, P2X3 receptor may become a new pharmacological target for relieving inflammatory pain.

Previous studies have shown that P2X3 could activate nuclear factor-kappaB (NF-κB) signaling pathway, regulate the level of inflammatory cytokines, and also cause extracellular signal-regulated kinases 1/2 (ERK1/2) phosphorylation, which can induce pain (Li et al., 2020; Varani et al., 2010). Meanwhile, the activation of ERK1/2 contributes to the germination and maintenance of P2X3-induced pain (Yu et al., 2013). These findings may bring new reference for investigation of the therapeutical agents of inflammatory pain.

Scutellaria baicalensis Georgi (SBG) is a perennial herb with traditional functions of clearing heat and dampness, purging fire and detoxification. It has anti-inflammatory, antibacterial, and antioxidant activities (Liu et al., 2022; Wang et al., 2018). SBG is traditionally used to treat inflammation of respiratory tract and gastrointestinal tract, abdominal cramps, bacterial and viral infections (Kim et al., 2009; Zhang et al., 2010). Clinically, SBG is commonly used for treating inflammatory diseases such as ulcerative colitis and arthritis (Cui et al., 2021; Wang et al., 2022). The ethanol extract of *Scutellaria baicalensis* Georgi (SGE) can enhance the immune response of Th1 cells and inhibit the immune response of Th2 and Th17 cells, and can also significantly

reduce inflammatory cell infiltration and inhibit the expression of iNOS and COX-2 (Jeong et al., 2011; Jin et al., 2019; Shin et al., 2014). It has been demonstrated that baicalin plays an analgesic role in bone cancer pain model, SNL model and inflammatory pain model, and baicalein could also alleviate cancer pain and neuropathic pain (Fang et al., 2020; Hu et al., 2015; Huang et al., 2020; Lai et al., 2018; Wang et al., 2021). However, the effects and mechanisms of SGE in relieving inflammatory pain have not been deeply studied.

CFA is an inflammatory agent containing inactivated mycobacterium tuberculosis, and it is normally used in inducing inflammatory pain in animal models (Yen et al., 2019). The objective of this research is to assess the analgesic potential of SGE in CFA-induced inflammatory pain rats, and the mechanism of SGE in alleviating inflammatory pain was explored by focusing on P2X3 receptor. The results may provide evidence for the analgesic effect of SGE, and targeting P2X3 receptor may be a novel direction for developing analgesic drugs.

2. Material and methods

2.1. Reagents and animals

Interleukin-1β (IL-1β, rat) kit, interleukin-6 (IL-6, rat) kit, tumor necrosis factor-α (TNF-α, rat) kit and BCA kit were purchased from Sangon Biotech (Shanghai, China). The anti-NF-κB, anti-P2X3, anti-COX-2, anti-ERK1/2, anti-p-ERK1/2 and anti-GAPDH were obtained from Proteintech Group Co., Ltd. (Chicago, IL), and the anti-p-NF-κB was supplied by Cell Signaling Technology (USA). The anti-rabbit lgG/HRP was supplied by Bioss Biotech (Beijing, China). The CFA was supplied by Sigma (USA). Traditional Chinese medicine *Scutellaria baicalensis* Georgi was purchased from Anhui Shenghaitang Pharmaceutical Co., Ltd (batch number 2020010371), and authenticated by Professor Xuemei Qin, Shanxi University. Plant samples were deposited at the Modern Research Centre of Chinese Medicine, Shanxi University (Taiyuan China), labeled as GL-2021-0201 (*Scutellaria baicalensis* Georgi). The aspirin (ASA) was obtained from Bayer Healthcare Company Ltd (Beijing, China), α, β-me ATP was obtained from Glpbio (USA). PrimeScript ™ RT Master Mix and TB

Green® Premix Ex Taq™ II were purchased from Takara Bio Inc (Japan). *Scutellaria baicalensis* Georgi was extracted twice with 10 times volume of 60% ethanol for 2 h. The filtrates and concentrate were combined, and the concentrated solution was freeze-dried. Therefore, ultra performance liquid chromatography-mass spectrometry (UPLC-MS) analysis was applied to conduct structural identification. The detailed methods for preparation and analysis of SGE were shown in the supplementary materials.

All animal experiment procedures were in accordance with NIH Guidelines for the Care and Use of Experimental Animals, and have been examined and approved by the Committee of Scientific Research in Shanxi University (Ethics number: SXULL2022062). Male Sprague-Dawley rats (180-220 g) were purchased from the Beijing Vital River Laboratories (Beijing, China). The rats were kept in captivity under standard experimental conditions (four rats per cage, temperature: 20-25 ℃, humidity: 30-60%, and light: 12 h light-dark cycle). Experimental rats could freely get food and water.

2.2. Animal experiments

2.2.1. Therapeutic evaluation of SGE on CFA-induced inflammatory pain rats.

The 72 rats were randomly partitioned into six groups (n = 12 per group): control group, CFA group, CFA plus SGE groups (1.5 g/kg, 3 g/kg, 6 g/kg), CFA plus aspirin (ASA) group (100 mg/kg). In CFA group, CFA plus SGE groups and CFA plus ASA group, 0.1 mL of CFA was injected into the right hind paw of each rat, then 0.1 mL of CFA was injected again one week later to ensure persistent inflammatory pain (Zhou et al., 2019). SGE and ASA were dissolved in 0.5% CMC-Na solution. After CFA injection (0th day), rats were administrated with SGE (1.5, 3 and 6 g/kg) (Xiao et al., 2020) or ASA (100 mg/kg) by gavage for 28 days. While rats in control group and CFA group were gavaged with 0.5% CMC-Na for 28 days. Behavioral tests were conducted on -7th day (before first injection), 0th day (after second injection), 7th, 14th, 21th and 28th day after administration of SGE (Fig. 1). Twenty-eight days after administration, the rats were killed and the tissue were collected for subsequent experiments.

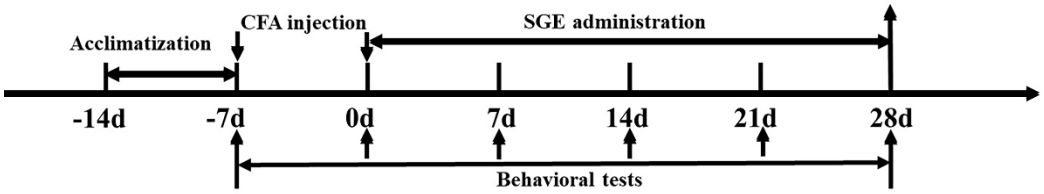

Fig. 1. Experimental procedure of CFA-induced inflammatory pain rats.

2.2.2. Therapeutic evaluation of SGE on inflammatory pain rats induced by CFA coupled with P2X3 agonist (α, β-me ATP)

The 48 rats were stochastically divided into four groups (n = 12): control group, CFA group, CFA plus α, β-me ATP group, CFA plus α, β-me ATP plus SGE (6 g/kg) group. 0.1 mL CFA was injected twice a week in the same manner. After CFA injection as aforementioned, rats were intragastrically administered with SGE or 0.5% CMC-Na for 28 days. Rats were administered with 0.1 mL α, β-me ATP (600 nmol, dissolved in sterile 0.9% saline solution) by intraplantar injection on 28th day. Behavioral tests were conducted on -7th day (before first injection), 0th day (after second injection), 7th, 14th, 21th and 28th day after administration of SGE, and 28th day after α, β-me ATP administration (Fig. 2). After the behavior tests, the rats were killed for subsequent experiments.

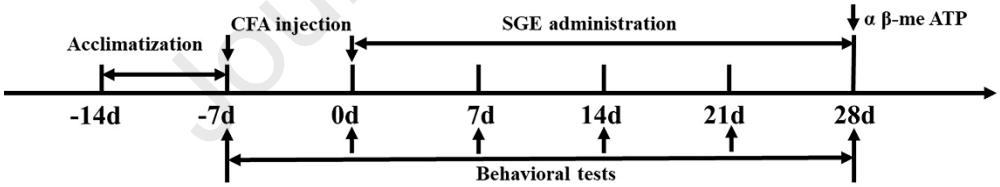

Fig. 2. Experimental scheme of inflammatory pain rats induced by CFA combined with α, β-me ATP.

2.3. Paw withdrawal threshold (PWT) test

Rats were put into a transparent plexiglass box and acclimated for 30 minutes. According to the method reported (Chaplan et al., 1994), von Frey hair with a series of pressures (0.4, 0.6, 1, 2, 4, 6, 8, 10, 15 and 26 g) was applied to the central surface of the right hind paw. If there was a positive reaction, the smaller force is used for stimulation; if not, the larger force in the series was applied for stimulation next. The PWT was calculated using the following formula: 50% PWT (g) = (10 [Xf+κδ]/10000),

198 "Xf" is the logarithm of the last von Frey hair in the sequence, "k" is the corresponding
199 value of the result sequence in the k-value table, and "δ" is the average difference
200 between stimuli (here, 0.224). If PWT > 26 g or < 4 g, values of 26 g and 4 g were
201 decided to be the maximum and minimum of PWT.

202 **2.4. Thermal withdrawal latency (TWL) test**

203 TWL was tested with Hargreaves's method (Yin et al., 2020). Briefly, rats were
204 placed on the surface of the Thermal Stimulator System (ZH-200, Anhui Zhenghua
205 Instrument Equipment Co., Ltd.), and adapted in a quiet environment for 15 min.
206 Infrared radiation was used to stimulate the right hind paw of rat. When rats raised their
207 hind paws, the lights were turned off, and the stimulation duration was recorded. The
208 tests were conducted for three times for each hind paw, with 5min interval. The cut off
209 time was set to 30 s to avoid burns. TWL were documented and the average value was
210 calculated for analysis.

211 **2.5. Paw withdrawal latency (PWL) test**

212 The thermal sensitivity was examined using the hot plate (Lee et al., 2015). Rats were
213 acclimatized for 5 min on the hot plate and the temperature was set at 52 ± 0.5 °C. The
214 latency period after the first paw lifting was recorded as the PWL, and three tests were
215 performed for each rat with an interval of 5 min. The cut off time was 30 s. PWL were
216 recorded and the average was calculated.

217 **2.6. Rotarod test**

218 Rotarod test was carried out using the described method (Rozas et al., 1997). The
219 rats were placed on the rotarod at 5 rpm for about 5 min 1 day before the test. The
220 rotation started at 5 rpm and continued to accelerate to the maximum of 25 rpm within
221 300 seconds. Rats were examined three times at 15-min intervals, and the fall latency
222 was recorded.

223 **2.7. Tissue preparation**

224 Rats were killed after behaviour tests. The right hind paw, and the L4-6 DRG of rats
225 were removed, quickly frozen in liquid nitrogen for subsequent experiments. In
226 addition, L4-6 DRG of three rats in each group were fixed in 4% paraformaldehyde

buffer for HE staining, Nissl staining or immunofluorescence experiment.

2.8. HE staining and Nissl staining

The fixed DRG was taken out for dehydration and waxed, and the waxed tissue was embedded in an embedding machine (JB-P5, China). The slices were cut into 4 μm sections with a paraffin microtome (RM 2016, China). After dehydration, the sections were stained with hematoxylin, and then stained with eosin dye for HE staining. The sections were dyed with toluidine blue for Nissl staining to observe the effects of SGE on the pathological changes of DRG.

2.9. Immunofluorescence

The fixed DRG was partitioned into 4 μm slices. After dewaxing and rehydration, antigen retrieval was performed. The slices were blocked with serum, incubated overnight with primary antibody (anti-P2X3, 1:50) at 4 ℃, then washed with PBS. Ultimately, slices were counterstained with DAPI and quenched by autofluorescence. Fluorescent Microscopy (Nikon, Japan) was used to capture the images, and Image-Pro Plus 6.0 software was used to analyze fluorescence intensity.

2.10. ELISA assay of inflammatory factors

Based on the instruction of ELISA kits, the levels of inflammatory factors (IL-6, IL-1β, TNF-α) in hind paw tissues of rats were detected. The OD values were recorded, and the concentrations of IL-6, IL-1β and TNF-α were calculated in accordance with the standard curve.

2.11. Western Blotting

In brief, L4-6 DRG of rats were dissolved in RIPA buffer (0.5 mL lysis buffer/100 mg protein). The concentration of protein was examined by BCA kit. Protein sample (40 μg protein/sample) was separated by electrophoresis, and then transferred to PVDF membrane. After being sealed with 5% non-fat milk for 2 h, the membrane was exposed to the antibody: GAPDH (1:2500), P2X3 (1:1000), COX-2 (1:1000), NF-κB (1:1000), p-NF-κB (1:1000), ERK1/2(1:1000), p-ERK1/2 (1:1000) and incubated overnight. After washing with TBST, the membrane was incubated with anti-rabbit lgG/HRP (1:5000). The protein signal was manifested by chemiluminescence.

2.12. qRT-PCR analysis

The mRNA levels of P2X3, COX-2, NF-κB, IL-1β, IL-6 and TNF-α in DRG of rats were examined by qRT-PCR. Primers were synthesized by Sangon Biotech (Shanghai, China) as shown in Table 1. Total RNA was isolated from rat DRG samples (6 samples in each group) and converted into cDNA by prime script RT Master Mix. TB Green® Premix Ex Taq™ II kit was used for amplification. The relative mRNA expression was calculated by using the $2^{-\Delta\Delta CT}$ method (Schmittgen et al., 2008).

Table 1

The sequences of primers applied in qRT-PCR.

Gene	Primer sequence forward (5′− 3′)	Primer sequence reverse (3′− 5′)
P2X3	GGAAACCTCCTGCCTAAC	TAACCACATCCCCTACCC
COX-2	GGGCAGGAAGTCTTTGGTCT	TTGGAACAGTCGCTCGTCAT
NF-KB	GCCTGACACCAGCATTTGA	CAAACCAAACAGCCTCACG
IL-1β	TGACGACCTGCTAGTGTGTG	TGGGTGTGCCGTCTTTCATC
IL-6	GCCTTCTTGGGACTGATG	TGGTCTGTTGTGGGTGGT
TNF-α	ACAAGGAGGAGAAGTTCCC	TCCGCTTGGTGGTTTGCTA
β-actin	TCAGGTCATCACTATCGGCA	GGCATAGAGGTCTTTACGGAT

2.13. Statistical Analysis

All data was represented as the mean ± SEM. The significant differences among the groups in behavioral experiments and other experiments were compared by ANOVA followed by Bonferroni's post hoc test. The value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Analysis of chemical ingredients in SGE by ultra performance liquid chromatography-mass spectrometry (UPLC-MS)

UPLC-MS can quickly and accurately identify the chemical components of SGE. By referring to our previous work (Gao et al., 2022;), literatures (Song et al., 2020; Zhao et al., 2018), and pubchem database (https://pubchem.ncbi.nlm.nih.gov/), a total of 19 chemical constituents were identified (Fig. S1, Table S1), including baicalin, baicalein,

wogonin and wogonin, etc.

3.2. SGE attenuates CFA-induced inflammatory pain behavior in rats

In order to explore whether SGE could restrain inflammatory pain, mechanical pain threshold, thermal pain threshold and motor coordination ability of rats were determined. The results revealed that PWT, TWL and PWL were obviously decreased after CFA injection as compared with control group. Compared with the CFA group, SGE (3 g/kg, 6 g/kg) significantly increased PWT on the 28th day, SGE (6 g/kg) markedly increased TWL on the 21th day, increased PWL and prolonged the latency time of the rotarod on 28th day. Meanwhile, the positive drug ASA (100 mg/kg) significantly increased PWT and TWL on the 21th day, and increased PWL and prolonged the latency time of the rotarod on 28th day as compared with CFA group (Fig. 3A-D). These results demonstrated that SGE could improve the mechanical pain threshold, the thermal pain threshold and the motor coordination ability of rats with inflammatory pain.

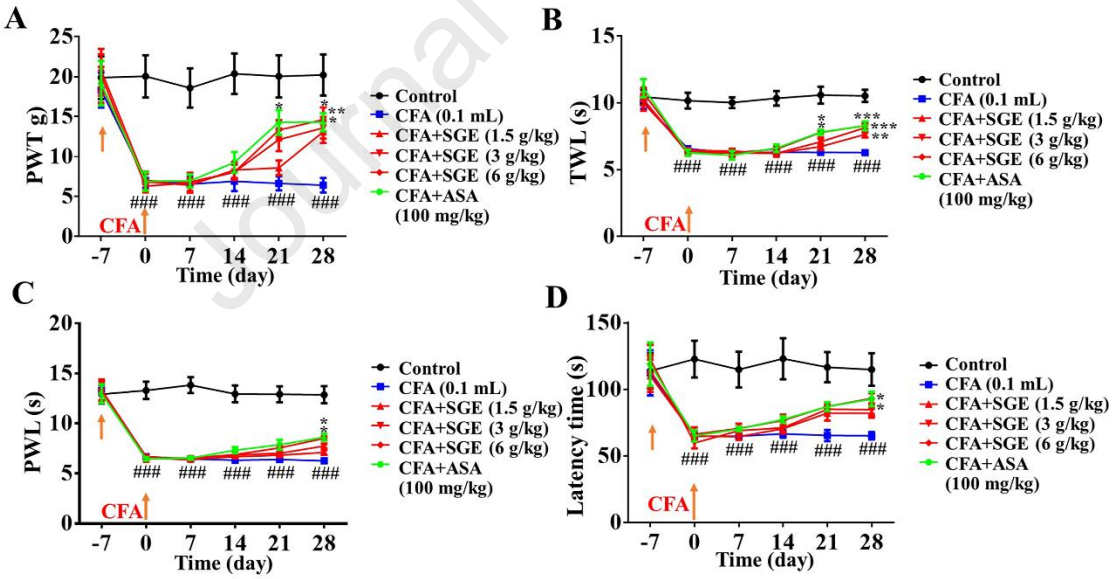

Fig. 3. SGE attenuates inflammatory pain behavior in CFA-induced rats. (A) paw withdrawal threshold test, (B) thermal withdrawal latency test, (C) paw withdrawal latency test, (D) rotarod test, (n = 9). $^{###}P < 0.001$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the CFA group.

3.3. SGE inhibits the pathological changes of dorsal root ganglion neurons in CFA-

induced rats.

HE staining and Nissl staining were used to determine the effects of SGE on the pathological alterations of DRG neurons in CFA-induced rats. The results revealed that DRG neurons were arranged neatly, stained evenly, and Nissl bodies were abundant, evenly distributed in the control group. After CFA injection, the DRG neurons were densely shrunk, arranged irregularly, and stained deeply and the number of Nissl bodies was notably reduced as compared with control group. While SGE (3 g/kg, 6 g/kg) treatment reduced the injury of DRG neurons (Fig. 4A), and increased the number of Nissl bodies as compared with CFA group (Fig. 4B). The results demonstrated that SGE had protective effects on DRG neurons of CFA-induced rats.

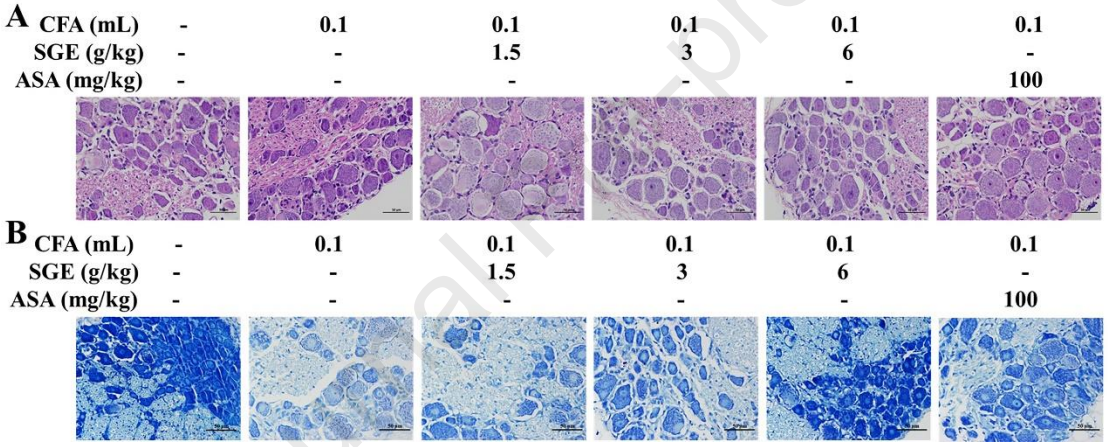

Fig. 4. The effects of SGE on pathological changes of DRG neurons in CFA-induced rats. (A) HE staining, (B)Nissl staining, Magnification: 400 times; Scale bar: 50 μm (n = 3).

3.4. SGE decreases the level of inflammatory cytokines in CFA-induced inflammatory pain rats.

To study the role of SGE on inflammatory pain, the levels of inflammatory cytokines (IL-1β, IL-6, TNF-α) in toe tissue of rats were determined. Compared with control group, the secretion of IL-1β, IL-6, TNF-α were augmented after CFA injection. Compared with CFA group, SGE (1.5 g/kg) could notably reduce the secretion of IL-1β, IL-6, TNF-α, SGE (3 g/kg) can markedly decrease the secretion of IL-1β, IL-6, while SGE (6 g/kg) could prominently reduce the secretion of IL-1β, TNF-α (Fig. 5A-C). These data indicated that SGE could reduce the secretion of inflammatory cytokines

in CFA-induced inflammatory pain rats.

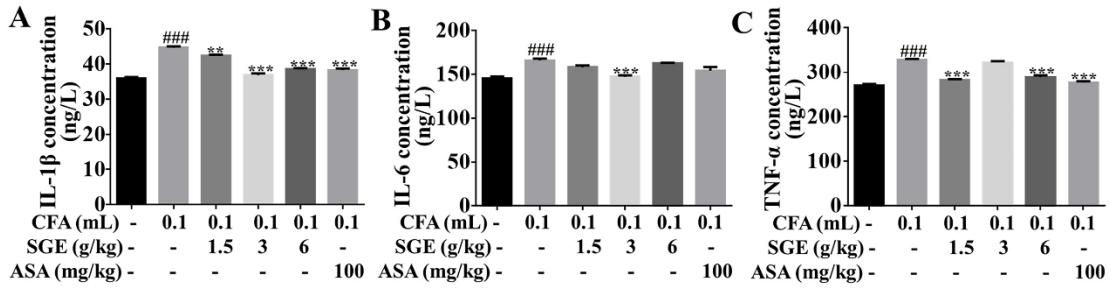

Fig. 5. Effect of SGE on inflammatory cytokines in toe tissue of CFA-induced rats. (A) IL-1β, (B) IL-6, (C) TNF-α, (n = 6). $^{###}P < 0.001$ vs. the control group; $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the CFA group.

3.5. Effects of SGE on the expression of NF-κB and COX-2 in DRG of CFA-induced rats.

The expressions of NF-κB and COX-2 are closely associated with inflammatory pain, and the release of COX-2 can be suppressed by restraining the activation of NF-κB (Ajayi et al., 2020). Western blot results suggested that compared with the control group, the phosphorylation of NF-κB and COX-2 in DRG were augmented. Compared with CFA group, SGE (6 g/kg) treatment could notably down-regulate the expression of the phosphorylation of NF-κB and COX-2 (Fig. 6A-D). Previous studies have demonstrated that P2X3 receptor could promote the activation of NF-κB (Varani et al., 2010), we wondered whether the inhibitory effect of SGE on NF-κB pathway was associated with the inhibition of P2X3.

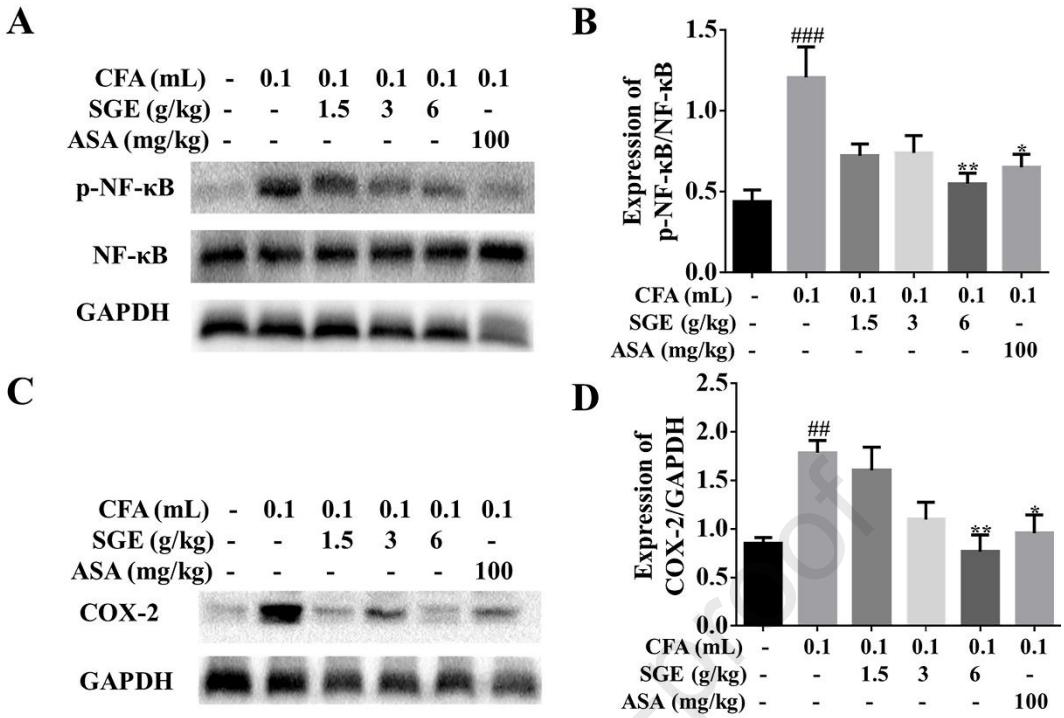

Fig. 6. SGE restrains the phosphorylation of NF-κB and COX-2 expression. (A-B) The expression of p-NF-κB; (C-D) The expression of COX-2, (n = 5). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the CFA group.

3.6. SGE suppresses P2X3 expression in dorsal root ganglion of CFA-induced rats.

Immunofluorescence and western blot were used to explore the influence of SGE on P2X3 expression in DRG of CFA-induced rats. The results revealed that the expression of P2X3 significantly increased after CFA injection as compared with the control group, while SGE (6 g/kg) treatment notably decreased the expression of P2X3 as compared with CFA group (Fig. 7A-D). The results demonstrated that SGE could notably decrease the elevation of P2X3 in DRG of CFA-induced rats.

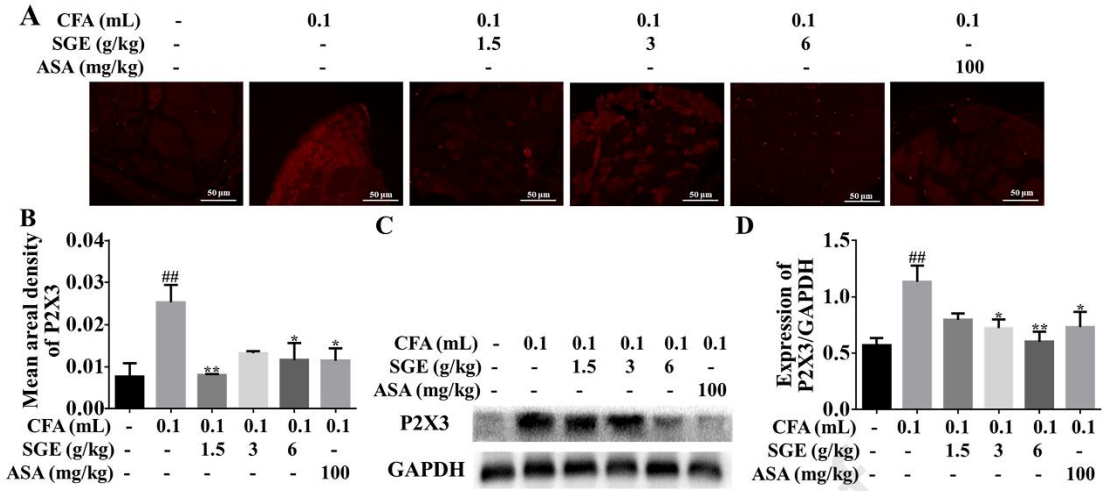

Fig. 7. Effect of SGE on P2X3 expression in DRG of CFA-induced rats. (A) Representative images of P2X3 expression in DRG detected by immunofluorescence. (B) Average intensity of P2X3 in DRG, Magnification: 400 times; Scale bar: 50 μm (n = 3). (C-D) The expression of P2X3 detected by western blot (n = 5). $^{\#\#}P < 0.01$ vs. the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the CFA group.

3.7. Effect of SGE on inflammatory pain behaviors induced by CFA coupled with P2X3 receptor agonist α, β me-ATP.

To explore whether P2X3 participates in the analgesic effect of SGE, α, β me-ATP, a P2X3 agonist, was injected into the right hind paw of CFA-induced rats. Compared with control group, PWT, PWL, TWL and latency times were markedly reduced after CFA injection, and the pain thresholds and motor coordination ability were further decreased upon administration of α, β me-ATP (600 nmol, 0.1 mL) on the 28th day. Compared with CFA plus α, β me-ATP group, SGE treatment significantly increased PWT, PWL, TWL and latency times (Fig. 8A-D). The results showed that SGE could reverse the decrease of mechanical pain threshold, thermal pain threshold and motor coordination ability caused by α, β me-ATP.

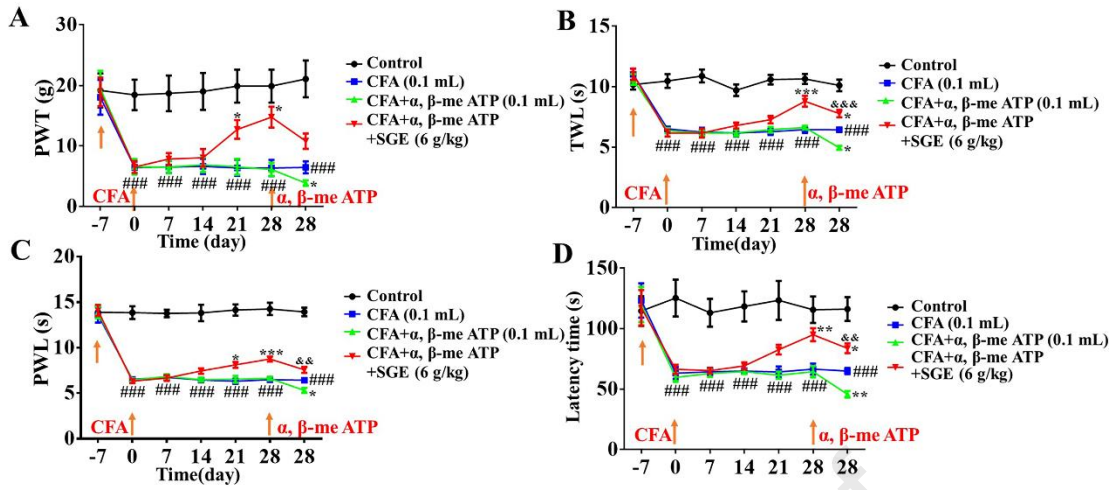

Fig. 8. Effects of SGE on behavioral changes induced by CFA coupled with P2X3 receptor agonist α, β me-ATP. (A) paw withdrawal threshold test, (B) thermal withdrawal latency test, (C) paw withdrawal latency test, (D) rotarod test, (n = 9). $^{###}P < 0.001$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the CFA group; $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ vs. the CFA plus α, β-me ATP group.

3.8. SGE alleviates the pathological changes of DRG and inhibits the elevation of P2X3 induced by α, β me-ATP.

To investigate the influence of SGE on the pathological changes of DRG after α, β me-ATP injection, HE staining was performed. The results showed that DRG neurons were irregular, dense and shriveled after CFA injection, and the DRG neurons were more seriously damaged in CFA plus α, β me-ATP group. However, compared with CFA plus α, β me-ATP group, the structure of neurons were close to normal, and the staining is becoming uniform in SGE treatment group (Fig. 9A).

The P2X3 expression in DRG was determined by immunofluorescence and western blot. Compared with control group, the mean intensity of P2X3 augmented after CFA injection. Compared with CFA group, the mean intensity was dramatically enhanced after α, β me-ATP injection. However, compared with CFA plus α, β me-ATP, the average intensity of P2X3 significantly decreased in SGE treatment group (Fig. 9B-C). Western blot revealed the similar results, which indicated that SGE could alleviate inflammatory pain by restraining P2X3 expression.

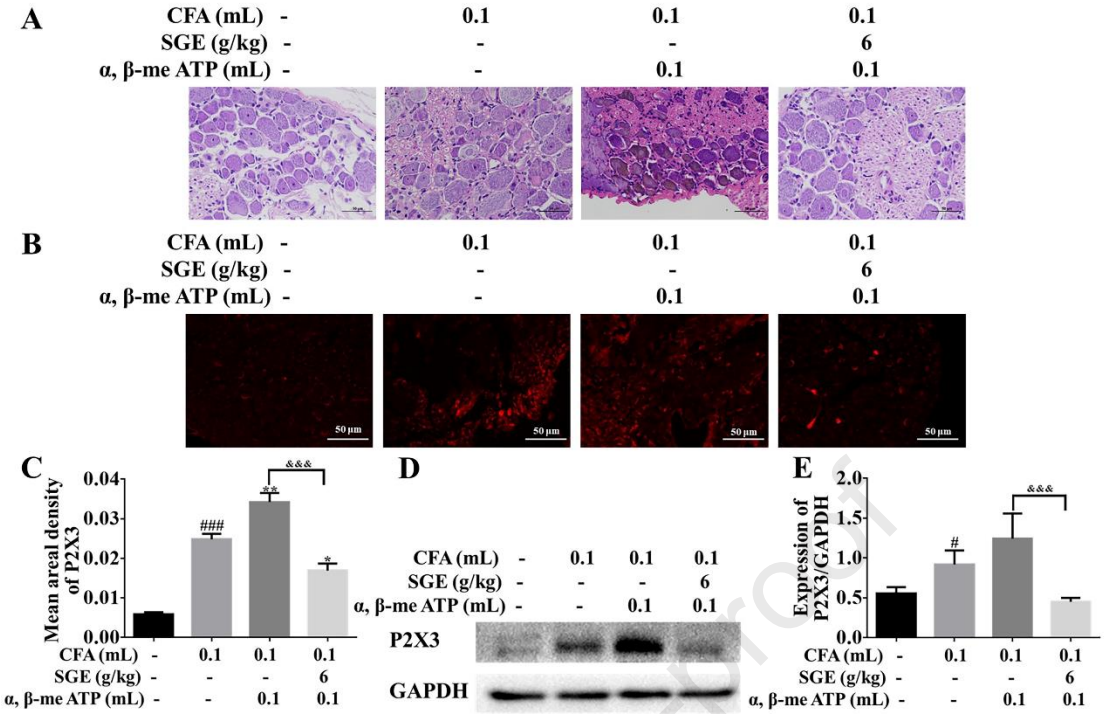

Fig. 9. Effects of SGE on pathological changes and P2X3 expression in DRG of rats induced by CFA coupled with α, β me-ATP. (A) The pathological changes of DRG were detected by HE staining, Magnification: 400 times; Scale bar: 50 μm (n = 3). (B-C) Representative images of P2X3 expression in DRG of rats detected by immunofluorescence, Magnification: 400 times; Scale bar: 50 μm (n=3). (D-E) P2X3 expression detected by western blot (n = 5). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the CFA group; $^{\&\&\&}P < 0.001$ vs. the CFA plus α, β-me ATP group.

3.9. Effect of SGE on inflammatory factors in rats induced by CFA coupled with α, β me-ATP.

To investigate whether SGE can repress the increase of inflammatory factors induced by α, β me-ATP, the levels of inflammatory factors (IL-1β, IL-6, TNF-α) in rat toe tissues were determined. The results demonstrated that the levels of IL-1β, IL-6 and TNF-α were augmented after CFA injection as compared with control group, and the secretion of IL-1β, IL-6 and TNF-α were notably increased further after α, β me-ATP injection. Compared with the CFA plus α, β me-ATP group, SGE treatment decreased the levels of IL-1β, IL-6 and TNF-α (Fig. 10A-C). The results indicated that SGE could

inhibit the levels of inflammatory factors by acting on P2X3 receptor.

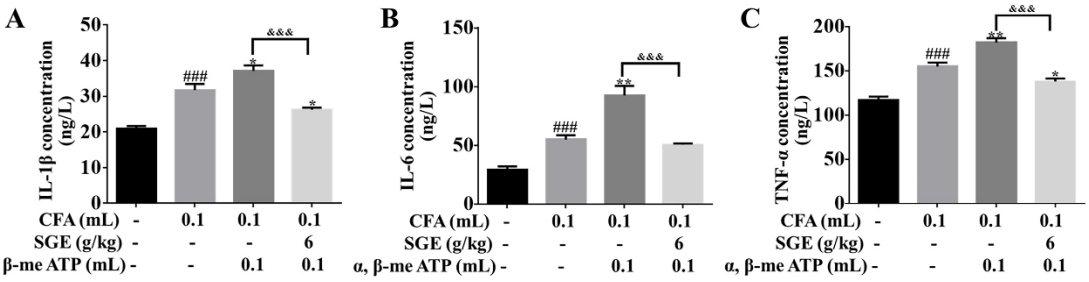

Fig. 10. Effect of SGE on inflammatory factors in toe tissue of inflammatory pain rats induced by CFA coupled with α, β me-ATP. (A) IL-1β, (B) IL-6, (C) TNF-α, (n = 6). $^{###}P < 0.001$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the CFA group; $^{\&\&\&}P < 0.001$ vs. the CFA plus α, β-me ATP group.

3.10. SGE inhibits NF-κB and ERK1/2 signaling pathways by acting on P2X3 receptor.

The above experiments have proved that SGE could play an analgesic role by repressing P2X3, and SGE could down-regulate the phosphorylation of NF-κB and COX-2 expression in DRG of rats. Related studies have shown that inhibiting P2X3 expression could down-regulate the phosphorylation of ERK1/2, thus alleviating mechanical hyperalgesia (Xiong et al., 2017). To verify whether SGE inhibits NF-κB and ERK1/2 pathway by acting on P2X3 receptor, western blot was performed. The results demonstrated that the protein levels of p-NF-κB, COX-2, and p-ERK1/2 were up-regulated after CFA injection as compared with control group, and the protein levels increased further after α, β me-ATP injection. However, compared with CFA plus α, β me-ATP group, the expression of p-NF-κB, COX-2, and p-ERK1/2 in SGE treatment group were down-regulated (Fig. 11A-C). The results demonstrated that SGE represses NF-κB and ERK1/2 signal pathways through restraining P2X3 receptor.

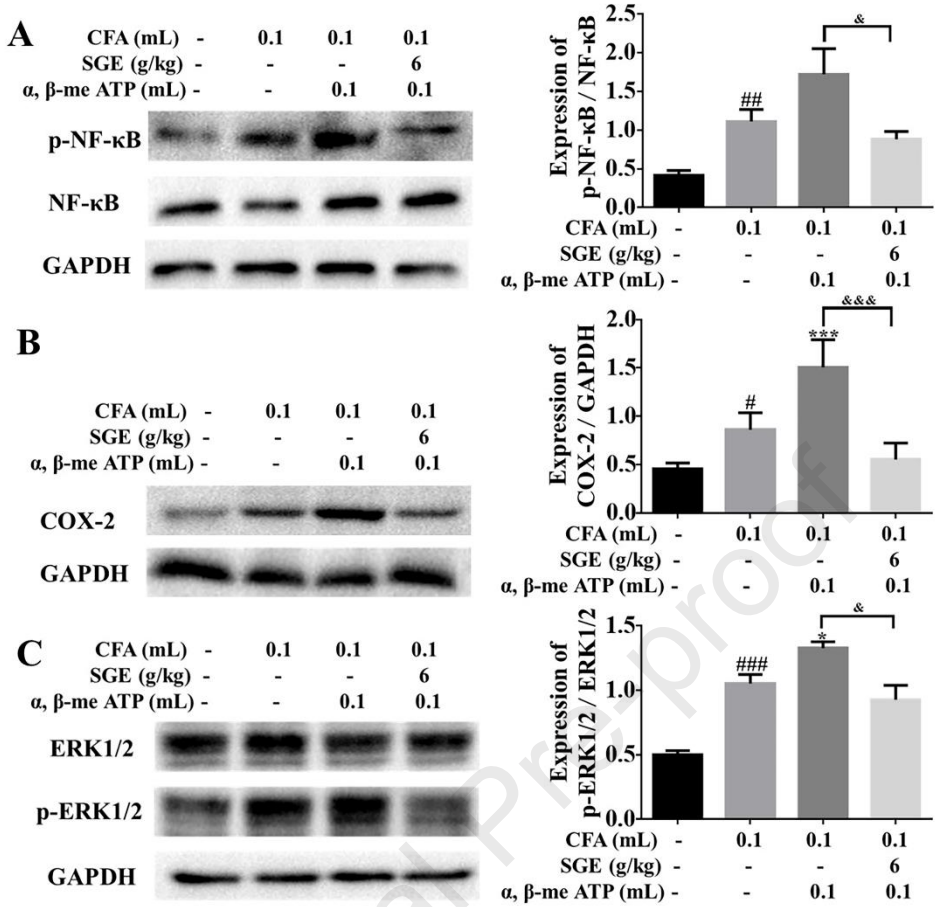

Fig. 11. Effects of SGE on the p-NF-κB, COX-2, p-ERK1/2 expression in DRG of rats induced by CFA coupled with α, β me-ATP. (A)The expression of p-NF-κB, (B) The expression of COX-2, (C) The expression of p-ERK1/2, (n = 5). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the control group; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. the CFA group; $^{\&}P < 0.05$, $^{\&\&\&}P < 0.001$ vs. the CFA plus α, β-me ATP group.

3.11. Effects of SGE on mRNA expression of P2X3 receptor-related genes in DRG of inflammatory pain rats

To explore the effects of SGE on mRNA expression levels of P2X3 receptor-related genes in DRG of inflammatory pain rats, qRT-PCR was performed. The results revealed that the mRNA expression levels of P2X3, COX-2, NF-κB, IL-1β, IL-6 and TNF-α in CFA group were markedly augmented as compared with control group, while the mRNA expression of these genes were significantly elevated after α, β me-ATP injection. However, compared with CFA plus α, β me-ATP group, the mRNA levels of these genes in SGE treatment group were notably decreased (Fig. 12 A-F). The results

indicated that SGE could downregulate the mRNA levels of P2X3, COX-2, NF-κB, IL-1β, IL-6 and TNF-α through restraining P2X3.

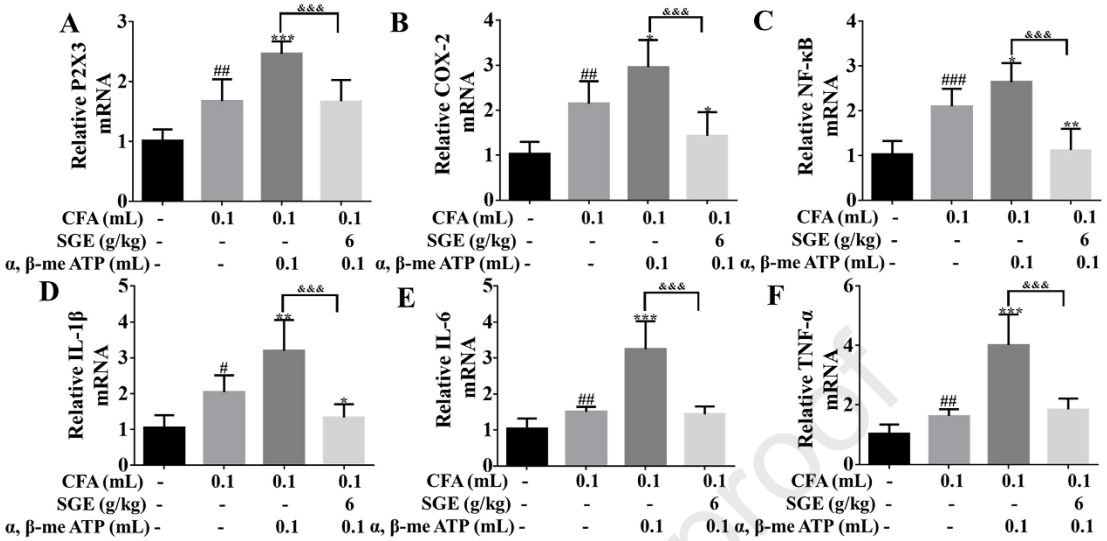

Fig. 12. The effects of SGE on mRNA expression levels of P2X3-related genes in DRG of inflammatory pain rats. (A) P2X3, (B) COX-2, (C) NF-κB, (D) IL-1β, (E) IL-6, (F) TNF-α, (n = 6). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the CFA group; $^{\&\&\&}P < 0.001$ vs. the CFA plus α, β-me ATP group.

4. Discussion

The prevalence of chronic pain has exceeded 35% in the world, which severely impacts the health of patients (Andrews et al., 2018). Inflammatory pain is a common chronic pain in clinical practice, which can significantly lower the pain threshold, including mechanical pain threshold and thermal pain threshold (Altarifi et al., 2019; Burek et al., 2021; Dougados et al., 2015; Li et al., 2021). The change of pain threshold is one of the important criteria for evaluating the curative effect of analgesic drugs.

In this work, a variety of methods were used to comprehensively evaluate the effects of SGE on behavioral changes of CFA-induced inflammatory pain rats. Particularly, Hargreaves test and hot plate test were both used for assaying thermal pain threshold, while differences were existed between the two methods. Hargreaves test could focus on the paws injected with CFA, however, the temperature was not definited in the test. In the hot plate test, all the paws of rats are exposed to thermal stimulation,

459 therefore the results may be affected by paws without CFA injection (Deuis et al., 2017;
460 Silva-Cardoso et al., 2023). Our results demonstrated that SGE could obviously
461 alleviate inflammatory pain by increasing the thermal pain threshold and mechanical
462 pain threshold. It has been known that the components of SGE could attenuate pain in
463 various models (Huang et al., 2020; Lai et al., 2018), however, the role of SGE in
464 alleviating inflammatory pain was a major finding in our study.

465 Moreover, our results indicated that SGE could reduce the secretion of
466 inflammatory factors including IL-1β, IL-6, TNF-α and down-regulate protein
467 expression of COX-2 and NF-κB. Studies have found that SBG extract and its effective
468 components could suppress the production of pro-inflammatory factors such as PGE2,
469 IL-5 by inhibiting the expression NF-κB (Chou et al., 2003; Kim et al., 2009; Kim et
470 al., 2010; Lee et al., 2015). Other studies reported that baicalin could improve
471 inflammatory pain by targeting Akt signal transduction (Fang et al., 2020), and
472 baicalein can relieve neuropathic pain by inhibiting the expression of proinflammatory
473 cytokines and reducing the activation of astrocytes (Lai et al., 2018), which were
474 consistent with our results.

475 P2X3 receptor participates in the pathological process of inflammatory pain and
476 neuropathic pain, and is regarded as a therapeutic target of chronic pain (Dong et al.,
477 2022). Studies have demonstrated gefapixant, a selective P2X3 receptor antagonist, has
478 therapeutic effect on chronic pain in patients with interstitial cystitis/bladder pain
479 syndrome, arthritis pain and endometriosis pain (Krajewski et al., 2020). Meanwhile,
480 the content of P2X3 is increased notably in DRG of CFA-induced inflammatory pain
481 model (Jiang et al., 2017). α, β-me ATP, a P2X3 receptor agonist, can cause hyperalgesia,
482 which was used to verify the potential role of SGE on P2X3 receptor (Lu et al., 2013).
483 Our results revealed that the pain thresholds of CFA-induced rats were further decreased
484 after administration of α, β-me ATP. However, SGE could reverse the action of α, β-me
485 ATP, raise the pain threshold of CFA-induced rats, alleviate pathological damage,
486 decrease P2X3 expression, and suppress the levels of inflammatory factors, indicating
487 that SGE could alleviate inflammatory pain by down-regulating the expression of P2X3.

Notably, it is an innovative discovery that SGE could suppress P2X3 expression, which is worth of further study. Interestingly, other studies also found several treatment approaches could relieve pain through reduction of P2X3 expression, including electroacupuncture stimulation and neferine administration (Dan et al., 2021; Xiang et al., 2019).

Researches has manifested that P2X3 can regulate NF-κB and ERK1/2 signaling pathways. NF-κB was a vital transcription factor, which controls the gene expression of nociceptive mediators and modulate pain by acting on inflammatory factors and COX-2 (Shih et al, 2015; Sun et al., 2012; Wang et al., 2011). It has been reported that P2X3 receptor can activate NF-κB (Varani et al., 2010). Conversely, NF-κB could also regulate P2X3 receptor in DRG, and their interaction mediates the up-regulation of P2X3 expression (Zhang et al., 2015; Zhou et al., 2015). ERK1/2 signaling pathway participates in inflammation, cell proliferation and differentiation, and is phosphorylated in response to harmful stimuli (Peti et al., 2013; Wanget al., 2014). The activation of P2X3 receptor is involved in phosphorylation of ERK1/2 in DRG neurons (Seino et al., 2006; Zhao et al., 2017). Our results demonstrated that SGE could reverse the phosphorylation of NF-κB and ERK1/2 induced by α, β me-ATP, and the RT-qPCR has also obtained similar results. Therefore, it is speculated that SGE may inhibit NF-κB and ERK 1/2 signal pathway by suppression of P2X3 receptor.

5. Conclusion

In summary, we found that SGE could increase the pain threshold of CFA-induced inflammatory pain rats, improve the pathological injury of DRG, and inhibit P2X3 expression. In addition, SGE could inhibit NF-κB and ERK1/2 signaling pathways and the secretion of pro-inflammatory cytokines by suppression of P2X3 receptors, thus alleviating inflammatory pain.

Author contributions

LG and J-XZ provided the concept and designed the study. J-XZ performed the experiments and wrote the draft of the manuscript. X-MQ, J-Z and LG provided

oversight. LG revised the manuscript and approved the manuscript submission. All authors read and approved the final manuscript. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Conflict of interest

The authors declare that they have no competing interests.

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Highlight

- The ethanol extract of *Scutellaria baicalensis* Georgi can alleviate complete Freund's adjuvant (CFA)-induced inflammatory pain.
- The ethanol extract of *Scutellaria baicalensis* Georgi alleviate inflammatory pain by down-regulating P2X3 expression.
- The ethanol extract of *Scutellaria baicalensis* Georgi can inhibit NF-κB and ERK1/2 activation by suppression of P2X3 receptor.

Conflict of interest

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All of the authors declared there are no conflicts of interest for this manuscript.