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Peripheral soluble epoxide hydrolase inhibition reduces hypernociception and inflammation in albumin-induced arthritis in temporomandibular joint of rats

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Abstract

Rheumatoid arthritis (RA) is characterized by chronic inflammation of the synovial tissue, joint dysfunction, and damage. Epoxyeicosatrienoic acids (EETs) are endogenous anti-inflammatory compounds, which are quickly converted by the soluble epoxide hydrolase (sEH) enzyme into a less active form with decreased biological effects. The inhibition of the sEH enzyme has been used as a strategy to lower nociception and inflammation. The goal of this study was to investigate whether the peripheral treatment with the sEH enzyme inhibitor 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU) could prevent the hypernociception and inflammation in the albumin-induced arthritis model in rats' temporomandibular joint (TMJ). After the induction of experimental arthritis, animals were assessed for nociceptive behavior test, leukocyte infiltration counts and histologic analysis, ELISA to quantify several cytokines and Western blotting. The peripheral pretreatment with TPPU inhibited the arthritis-induced TMJ hypernociception and leukocyte migration. Moreover, the local concentrations of proinflammatory cytokines were diminished by TPPU, while the anti-inflammatory cytokine interleukin-10 was up-regulated in the TMJ tissue. Finally, TPPU significantly decreased protein expression of iNOS, while did not alter the expression of MRC1. This study provides evidence that the peripheral administration of TPPU reduces hypernociception and inflammation in TMJ experimental arthritis.

Keywords

Soluble epoxide hydrolase enzyme; TPPU; Temporomandibular joint; Hypernociception; Inflammation

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune systemic and inflammatory disease, which can cause damage to different organs and tissues, but predominantly affects the joints [1,2]. RA is characterized by activation of resident synovial inflammatory cells, mainly macrophages, leukocyte infiltration in the synovium and joint cavity, and the production of an inflammatory milieu consisting of proinflammatory cytokines and chemokines, which in turn contribute to synovial membrane hyperplasia, characterizing the rheumatoid *pannus* [1,2]. The involvement of the temporomandibular joint (TMJ) in patients with RA has a prevalence ranging from 65 to 92% [3,4] and the commonly reported symptoms include pain in the TMJ region and masticatory muscles, joint stiffness and limited function [5]. Severe RA disease stages in the TMJ may lead to sequelae and disabilities; therefore, the early diagnosis and appropriate treatment and management are warranted [6].

Epoxyeicosatrienoic acids (EETs) are eicosanoids derived from the metabolism of arachidonic acid by cytochrome P450 (CYP450) enzymes. Arachidonic Acid is metabolized to four biologically active EET regioisomers, the 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET [7]. All EETs are then further metabolized into Jess active dihydroxy-eicosatrienoic acids (DHETs) by the enzyme soluble epoxide hydrolase [8]. Consequently, the activity of the enzyme sEH is considered one of the main determinants of the bioavailability of EETs. Biologically, studies have been shown that the EETs reduce the inflammation [9,10], and have analgesic, antifibrotic, and antihypertensive effects, acting in both paracrine and autocrine fashion [11–15].

The sEH inhibitors can act in synergy with existing anti-inflammatory drugs, including cyclooxygenases (COX) and lipoxygenases (LOX) inhibitors [16–18], as well as anti-inflammatory phosphodiesterase inhibitors [12]. Thus, sEH inhibitors arouse great interest for their therapeutic approach, since their use increases the *in vivo* concentration of EETs and other fatty acids, resulting in anti-inflammatory, antinociceptive, antihypertensive, neuroprotective, and cardioprotective activity [19–24].

The therapy for RA is based on two principal approaches: symptomatic treatment with non-steroidal anti-inflammatory drugs and disease-modifying anti-rheumatic drugs (DMARDs) [25,26]. Among DMARDs, the methotrexate (MTX) is the standard first-line pharmacotherapy for RA, although about 40% of RA patients are unresponsive to MTX treatment [27]. Thus, the literature highlights the necessity of new therapeutic targets for the treatment of RA. The possibility to locally administer the drug is of great interest in order to have a local effect and without any systemic effect. The current study tested the peripheral anti-hypernociceptive and immunomodulatory effects of the sEH inhibitor TPPU in the albumin-induced arthritis model in rats' TMJ.

2. Materials and methods

2.1. Subjects and general procedures

In this study, we used male Wistar rats (7 weeks old, 200–240 g) obtained from ANILAB (Animais de Laboratório - Criação e Comércio Ltda, Paulinia, SP, Brazil). The rats were

housed in plastic cages with soft bedding (four/cage) on a 12:12 light cycle (lights on at 06:00 AM) with food and water available *ad libitum*. They were maintained in a temperature-controlled room (± 23 °C) and handled for at least one week prior to the experiments. Experimental protocols were approved by the Committee on Animal Research of the Faculdade São Leopoldo Mandic (CEUA/SLMANDIC #2018/016) and were carried out following the guidelines of the National Council for Control of Animal Experimentation (CONCEA) and ARRIVES guidelines [28]. Each animal was used once and the number of animals per group was kept to a minimum. The group size (n) for each experimental group was described in each figure legends. Animals were divided randomly into experimental groups and used once. All of the observations, experimental data collection, and primary data analysis were conducted in a fashion where the treatments given to the experimental animals were blinded.

2.2. Induction of experimental arthritis

The protocol used to induce the experimental arthritis was described previously [29]. Briefly, at day "0" male Wistar rats were sensitized with 500 μg of methylated bovine serum albumin (mBSA) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 200 μL of an emulsion containing 100 μL phosphate-buffered saline (PBS) and 100 μL Freund's complete adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA) administered by subcutaneous injection in the back of the rat. Booster injections of mBSA dissolved in Freund's incomplete adjuvant (IFA) (Sigma-Aldrich, St. Louis, MO, USA) were given 7 and 14 days after the first immunization in different sites in the back. Non-immunized rats (control group) received similar subcutaneous injections but without the antigen (mBSA). Twenty-one days after the initial injection, TMJ arthritis was induced in the immunized animals by the intra-TMJ injection of mBSA (10 μg/TMJ) dissolved in 15 μL of PBS (challenge). Both non-immunized rats (control group) and immunized rats were challenged with an intra-TMJ injection of mBSA (10 μg/TMJ). Twenty-four h later, both non-immunized rats (control group) and immunized rats (arthritis-induced groups) received an intra-TMJ injection of a low dose of formalin (0.5%). The experimental design is summarized in Fig. 1.

The albumin-induced arthritis is an experimental model of delayed-type hypersensitivity (DTH) with mBSA as antigen. DTH is an inflammatory reaction mediated by effector memory T lymphocytes that infiltrate the site of injection of an antigen against, which the immune system has been primed. Once the rats received just one intra-TMJ injection of mBSA (challenge), this model induced an acute response 24 h after the challenge [29,30].

2.3. General procedures

Testing sessions took place during the light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23 °C. Each animal was placed in a mirrored wood test chamber (30 × 30 × 30 cm) with a glass at the front side, for a 15-min habituation period to minimize stress. Each animal was removed from the test chamber and briefly anesthetized by inhalation of isoflurane (2%) to allow the TMJ injections.

2.4. Temporomandibular joint (TMJ) injections

The TMJ injections were performed according to detailed procedures described in detail elsewhere [31]. Briefly, a 30-gauge needle connected to a cannula consisting of a polyethylene tube (P20) and to a Hamilton syringe (50 μL) was introduced into the left posteroinferior border of the zygomatic arch and advanced in an anterior direction until reaching the posterolateral aspect of the condyle of the TMJ. The total volume per injection was 30 μL.

2.5. Drugs and doses

The following drugs were used: 0.5% formalin solution prepared from commercially available stock formalin (aqueous solution of 37% of formaldehyde, Sigma-Aldrich, St. Louis, MO, USA) [29] and the selective soluble epoxide hydrolase (sEH) inhibitor, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU, 30 ng/TMJ). The formalin solution was diluted in 0.9% NaCl. TPPU was kindly provided by Dr. Bruce Hammock (University of California, Davis, California, USA) and dissolved in polyethylene glycol (PEG400).

2.6. Effect of the TPPU in the hypernociception of albumin-induced arthritis in TMJ of rats

To assess the arthritis-induced TMJ inflammatory hypernociception in rats, we applied a low dose of formalin (0.5%, 24 h later) into TMJ challenged by an intra-TMJ injection of mBSA, as previously described [29,32].

To test the effect of the selective sEH enzyme inhibitor, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU) on arthritis-induced TMJ hypernociception, the rats were pretreated (15 min prior formalin) with an intra-TMJ injection of TPPU (30 ng/TMJ) or its vehicle (Polyethylene glycol, PEG400, Sigma-Aldrich, St. Louis, MO, USA).

2.7. Measurement of behavioral nociceptive responses

Immediately after the 0.5% formalin TMJ injection, the animals recovered from anesthesia and immediately returned to the test chamber for a 45 min observation period of the behavioral nociceptive response. The time evaluation was divided into 9 × 5 min blocks. For each block of 5 min, the nociceptive score was determined measuring the number of seconds of two types of nociceptive behavior: 1) rubbing the orofacial region asymmetrically with the ipsilateral fore and hind paw, which was quantified by the amount of time that the animal exhibited it (using a chronometer) and/or 2) flinching the head in an intermittent and reflexive way characterized by high-frequency shakes of the head, which was quantified by its occurrence (using a cell counter). Since head flinches followed a uniform pattern of 1 s of duration, each flinch was expressed as 1 s. Results are expressed as the duration time of nociceptive behavior [33]. The sum of these nociceptive behaviors (rubbing the orofacial region + flinching of head) was used as a quantitative measurement of TMJ hypernociception. During the tests, the animals had no access to water or food.

Immediately after the analysis of the nociceptive responses, rats were killed by cervical dislocation under deep anesthesia (80 mg/kg ketamine and 20 mg/kg xylazine, intraperitoneally [i.p.]) and the periarticular tissues were dissected.

2.8. Periarticular tissue dissection

Periarticular tissue was isolated, as described before [34]. In brief, the skin covering the TMJ was removed and discarded. Temporalis and posterior deep masseter muscles were carefully dissected with careful attention to anatomic landmarks (zygomatic arch and tympanic bulla) until exposure of the condylar process. The samples included all of the tissues surrounding the condylar process, including the masticatory muscles (temporalis, posterior deep masseter, and pterygoideus externus), articular cartilage, fibrocartilage of the disc, and lateral ligaments. The standard sample size was 1 × 1 × 0.5 cm, as previously standardized [34].

2.9. Leukocyte infiltration analysis

Immediately after the nociceptive responses analysis and periarticular tissues dissection, the TMJ cavity was washed with 10 μL of PBS/EDTA (1 mM) for leukocyte infiltration analysis, as previously described [30]. Briefly, total leukocyte counts were realized in a Neubauer chamber diluting the exudate in the Türk solution (1:2). The differential leukocyte counts were performed by preparing smears in a cytocentrifuge, which were stained with a Fast Panoptic kit (Laborclin Ltda, Pinhais, PR, Brazil). An optical microscope (1000 × increase) was utilized to count the differential cells (100 cells total). The result of each cell type was calculated using the percentage of those cells and the total number of cells obtained in the total count. The results were expressed as the number of cells × 10^4/cavity.

2.10. Tissue preparation and histological analysis and score of TMJ

Rats of a new set of experiments, immediately after TMJ treatments, were terminally anesthetized with ketamine (85 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and perfused through the ascending aorta with 0.9% NaCl solution followed by 4% paraformaldehyde (PFA, pH 7.4, 4 °C). After perfusion, the head of the rat was removed, fixed in 10% buffered neutral formalin for 48 h, and decalcified for eight weeks with 10% ethylenediaminetetraacetic acid (EDTA) in 0.01 M phosphate-buffered saline (PBS) at 4 °C with three fresh solution changes per week. After that, TMJs were removed in a block and the decalcified samples were briefly washed in running tap water, dehydrated, and embedded in paraffin wax. Each sample was sliced into 6 μm sections in the sagittal plane and staining with hematoxylin-eosin (H&E). The sections were examined and scored using light microscopy, according to inflammatory cell infiltrate intensity, by an examiner blinded to the experimental conditions. The protocol used to score the sections was adapted from the previous study [35], The score of inflammatory infiltrate was evaluated in 5 rats and classified on a qualitative scale of 0–5 as follows: 0 (absence of inflammatory cells); 1 (1 – 10% of inflammatory cells); 2(11 – 25% of inflammatory cells); 3 (26 – 50% of inflammatory cells); 4 (51 – 75% of inflammatory cells) and 5 (> 75% of inflammatory cells). The final score was determined by adding the scores above for each of the samples.

2.11. Enzyme-Linked immunosorbent assay (ELISA) procedure

To study the effect of TPPU in the concentration of proinflammatory and anti-inflammatory cytokines concentration (TNF-α, IL-1β, IL-6, CINC-1, IL-10, IL-17, IL-23, and IFN-γ) on TMJ periarticular tissue, the ELISA assay was performed [33]. Each experimental group

was conducted on six different animals. The periarticular tissues sample from each animal were weighed and homogenized separately in the same weight/volume proportion in buffer with protease inhibitors (Ripa Lysis Buffer, Santa Cruz, Biotechnology, Dallas, Texas, USA), followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatants were stored at −80 °C until further analysis. The inflammatory cytokines were quantified by the following ELISA kits: TNF-α, Rat TNF-α/TNFSF1A DuoSet ELISA Kit (R&D Systems, Minneapolis, MN, USA, #DY510); IL-1β, Rat IL-1β/IL-1F2 DuoSet ELISA Kit (R&D Systems, Minneapolis, MN, USA, #DY501); IL-6, Rat IL-6 DuoSet ELISA Kit (R&D Systems, Minneapolis, MN, USA, # DY506); CINC-1, Rat CXCL1/CINC-1 DuoSet ELISA Kit (R&D Systems, Minneapolis, MN, USA, #DY515); IL-10, Rat IL-10 DuoSet ELISA Kit (R&D Systems, Minneapolis, MN, USA, #DY522); IFN-γ, Rat IFN-gamma DuoSet ELISA Kit (R&D Systems, Minneapolis, MN, USA, #DY585); IL-17, Rat IL-17A ELISA MAX™ Deluxe Set (BioLegend, San Diego, CA, USA, #437904); IL-23, RayBio® Rat IL-23 ELISA Kit (RayBiotech, Peachtree Comers, GA, USA, #ELR-IL23). All samples were tested in a duplicate manner according to the manufacturer's instructions, to guarantee the accuracy of the results.

2.12. Western blotting

The total protein yield in the TMJ periarticular tissues was measured through the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Protein samples (80 μg) of periarticular tissues were separated on SDS/PAGE gel and transferred for nitrocellulose membranes. A molecular mass standard (Bio-Rad, Hercules, CA, USA) was run in parallel to estimate molecular mass. The blockade of the membranes was performed in TBST (Tris-Buffered Saline, 0.1% Tween 20) containing 5% of nonfat milk at 4 °C overnight. The membranes were washed in TBS and then incubated at room temperature with primary antibodies: iNOS (Ml macrophage) antibody (overnight, 1:500, #18985–1-AP, Proteintech, Rosemont, IL, USA), MRC1 (M2 macrophage) antibody (overnight, 1:500, #18704–1-A, Proteintech, Rosemont, IL, USA) or GAPDH antibody (2h, 1:1000, #5174, Cell Signaling Technology, Danvers, MA, USA), used as a housekeeping protein. The membranes were then rewashed and incubated (2 h) with the appropriate secondary antibody conjugated with peroxidase (1:10000, #A6667, Sigma-Aldrich, St. Louis, MO, USA). The target protein was visualized in the membrane using a chemiluminescence-based ECL system (Amersham Biosciences, Piscataway, NJ, USA) and the digital image was obtained by CCD camera imaging for chemiluminescence (ImageQuant LAS 4000 mini, GE Healthcare Life Sciences, Pittsburgh, PA, USA). The program Image J (National Institutes of Health, Bethesda, USA) was utilized to measure the optical density of the bands.

2.13. Statistical analysis

To determine if there were significant differences ($P < 0.05$) between treatment groups, One-way ANOVA or T-test was performed. If there was a significant between-subjects main effect of treatment group following One-way ANOVA, post-hoc contrasts using the Tukey test were used to determine the basis of the significant difference. All data were tested for normality before statistics tests. Data are presented as means ± standard deviation.

3. Results

3.1. TPPU inhibits the arthritis-induced TMJ hypernociception

The albumin-induced arthritis elicited nociceptive behavioral responses in immunized rats (arthritis-induced group), but not in non-immunized rats (control group, Fig. 2, $p < 0.05$, one-way ANOVA, Tukey test). The pretreatment with the selective sEH inhibitor, TPPU (30 ng/TMJ, i.a., 15 min prior formalin), but not with its vehicle (Polyethylene glycol, PEG400, i.a., 15 min prior formalin), prevented the arthritis-induced TMJ hypernociception in immunized rats (Fig. 2, $p < 0.05$, one-way ANOVA, Tukey test).

3.2. TPPU reduces the arthritis-induced leukocyte migration in the TMJ

The albumin-induced arthritis evoked a significant leukocyte migration in the TMJ of in immunized rats (arthritis-induced group), but not in non-immunized rats (control group, Fig. 3A, $p < 0.05$, one-way ANOVA, Tukey test). These exacerbated leukocytes exudate was characterized by lymphocytes (Fig. 3B), neutrophils (Fig. 3C), mast cells (Fig. 3D), and macrophages (Fig. 3E). The pretreatment with TPPU (30 ng/TMJ, i.a.), but not with its vehicle (PEG400, i.a.), in immunized rats significantly reduced the arthritis-induced migration of total leukocytes (Fig. 3A), lymphocytes (Fig. 3B), neutrophils (Fig. 3C), and mast cells (Fig. 3D) in the TMJ ($p < 0.05$, one-way ANOVA, Tukey test). The number of macrophage cells was not affected by the pretreatment with TPPU (30 ng/TMJ) ($p > 0.05$, one-way ANOVA, Tukey test).

Histological analysis and score of the TMJ confirm these results (Fig. 4), showing no leukocyte influx in the non-immunized group (control group, Fig. 4A, B, and G) besides an important inflammatory cells influx in joint space in immunized rats (arthritis-induced group, Fig. 4C, D, and G), as indicated by red arrows. The pretreatment with TPPU (30 ng/TMJ,) significantly reduced ($p < 0.05$, one-way ANOVA, Tukey test) the arthritis-induced histological changes (Fig. 4E, F, and G).

3.3. TPPU modulates the concentration of inflammatory cytokines in arthritis-induced in the TMJ

The albumin-induced arthritis significantly increased the concentration of the proinflammatory cytokines TNF-α (Fig. 5A), IL-1β (Fig. 5B), IL-6 (Fig. 5C), CINC-1 (Fig. 5D), IL-17 (Fig. 5E), IL-23 (Fig. 5F), and IFN-γ (Fig. 5G) in the TMJ of immunized rats (arthritis-induced group) when compared with the non-immunized rats (control group) ($p < 0.05$, one-way ANOVA, post hoc Tukey test). The pretreatment with local administration of TPPU (30 ng/TMJ, i.a) in immunized rats abrogated the arthritis-induced amount of the proinflammatory cytokines TNF-α (Fig. 5A), IL-1β (Fig. 5B), IL-6 (Fig. 5C), CINC-1 (Fig. 5D), IL-17 (Fig. 5E), IL-23 (Fig. 5F), and IFN-γ (Fig. 5G) in the TMJ periarticular tissue ($p < 0.05$, one-way ANOVA, Tukey test).

The albumin-induced arthritis did not alter the amounts of the anti-inflammatory cytokine IL-10 (Fig. 5H) in the TMJ periarticular tissue of the immunized rats (arthritis-induced group) when compared with the non-immunized rats (control group) ($p > 0.05$, one-way ANOVA, post hoc Tukey test). On the other hand, the pretreatment with TPPU (30 ng/TMJ,

i.a.) in immunized rats significantly increased the concentration of IL-10 (Fig. 5H) in the TMJ periarticular tissue ($p < 0.05$, one-way ANOVA, Tukey test).

3.4. TPPU down-regulates the expression of the inducible nitric oxide synthase (iNOS) in the TMJ tissue

Western blotting analysis demonstrated that albumin-induced arthritis did not alter the expression of inducible nitric oxide synthase (iNOS, Fig. 6A) and C-type mannose receptor 1 (MCR1, Fig. 6B) in the TMJ periarticular tissue of the immunized rats (arthritis-induced group) when compared with the non-immunized rats (control group) ($p > 0.05$, one-way ANOVA, post hoc Tukey test). The pretreatment with TPPU (30 ng/TMJ, i.a.) in immunized rats significantly decreased the expression of iNOS (Fig. 6A, $p < 0.05$, one-way ANOVA, Tukey test) in the TMJ periarticular tissue. On the other hand, there was no difference among experimental groups in the expression of MCR1 (Fig. 6B, $p > 0.05$, one-way ANOVA, Tukey test).

4. Discussion

In the present study, we have demonstrated that albumin-induced arthritis in the rats' TMJ evoked hypernociception, leukocyte accumulation, and increase the concentration of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, CINC-1, IL-17, IL-23, and IFN-γ, corroborating with the data previously obtained by our research group [29,30]. In addition, we showed that the peripheral pretreatment with an intra-TMJ injection of the sEH enzyme inhibitor, TPPU, inhibited the arthritis-induced TMJ hypernociception and inflammation. These effects were associated with the decrease of proinflammatory cytokines release, the reduction of inflammatory cells infiltrate in the TMJ tissue, as well as by down-modulating the inducible nitric oxide synthase (iNOS) expression, a hallmark molecule of M1 macrophages [36].

These findings are of clinical relevance because joint inflammation (e.g., synovitis) plays a crucial role in the development and progression of RA [37] as well as in several clinical symptoms, such as articular pain [38]. Synovial fibroblasts and activated immune cells are responsible for the production of proinflammatory cytokines, which in turn contribute to synovitis, leukocyte accumulation, and consequently to nociceptor sensitization in the joints [39]. In fact, in patients with joint disease [40] and in experimental arthritis [29,33,41], the concentration of proinflammatory cytokines such as TNF-α, IL-lβ, IL-6, IL-17, IFNγ, and CINC-1 is increased in the synovial fluid, as well as the leukocyte accumulation [42]. In agreement with our findings, it has been shown that the inhibition of sEH may have a role in decreasing inflammatory joint pain in a synovitis model in horses [43].

While it has been suggested that the epoxyeicosatrienoic acids (EETs) did not reduce leukocyte recruitment in tobacco smoke-induced lung inflammation rat model [44], another study has demonstrated that one of the anti-inflammatory effects of the EETs is to reduce nuclear factor kappa B (NF-κB) signaling, leading to a reduction in adhesion molecule expression and, thus, leukocyte recruitment [45]. Moreover, the inhibition of sEH reduced the recruitment of eosinophils and mast cells to the intestinal mucosa in a murine model of food allergy [46]. Corroborating, in this present study, we demonstrated that the peripheral

anti-inflammatory effects of sEH inhibitor TPPU are dependent, at least in part, of leukocyte recruitment inhibition, since the pretreatment with TPPU significantly reduced the lymphocytes, neutrophils and mast cells migration to the albumin-induced arthritis in the TMJ of rats.

Our results demonstrated that the inhibition of sEH by TPPU reduced the increased amount of the proinflammatory cytokines TNF-α, IL-1β, IL-6, CINC-1, IL-17, IL-23, and IFN-γ in the albumin-induced arthritis model in the TMJ of rats. Those inflammatory cytokines are considered key mediators in joint inflammation and bone destruction during RA. For example, TNF-α regulates the formation of other inflammatory mediators in the synovial tissue, such as IL-1β, IL-6, and IL-8 [47], which in turn induce cell proliferation [48], endothelial activation, neutrophil chemotaxis [49], and an influx of polymorphonuclear cells into the joint [50]. IFN-γ is an important proinflammatory cytokine promoting disease severity in RA experimental models [51–54]. Studies have shown that interaction between IL-23 and IL-17 is not only essential for the onset phase, but also for the destruction phase of RA [55], promoting the release of proinflammatory mediators in the diseased synovium [56]. This milieu of cytokines is crucial to mediate inflammatory and neuropathic pain [57], linking the inflammatory stimuli and the release of final mediators (as the prostaglandins and sympathomimetic amines), which in turn, directly sensitize the primary afferent nociceptors [58,59].

We have recently shown that the systemic treatment with TPPU decreased the expression of several inflammatory genes in the knee joint of mice in collagen-induced arthritis (CIA) model, such as IFN-γ, IL-17, IL-23, TNF-α, IL-1β, and IL-6 [60]. Taken together, these findings reinforce our suggestion that the inhibition of the sEH enzyme, and consequently, a possible increase in the bioavailability of EETs in the tissue, have analgesic and anti-inflammatory effects in the antigen-induced RA model in the TMJ of rats. The increase in the bioavailability of EETs seems to promote peripheral immunomodulation in albumin-induced arthritis in the TMJ, reducing the infiltration of inflammatory cells and the release of the key RA proinflammatory cytokines into the TMJ tissue and consequently reducing hypernociception. It has been demonstrated that the sEH protein expression levels were increased in CIA animals, and the systemic treatment with TPPU decreased the protein levels of sEH in the knee joint of CIA animals [60] and even though this parameter has not been evaluated in the current study, the same could occur in the TMJ of rats, a hypothesis that warrants further testing.

On the other hand, our present results showed that the pretreatment with TPPU increases the levels of the anti-inflammatory cytokine interleukin-10 (IL-10) in the TMJ tissue. The cytokine IL-10 plays a therapeutic role in various animal models of arthritis, since it inhibits the production and effects caused by proinflammatory cytokines, reducing inflammation, cellular infiltrates, and joint destruction [61,62]. IL-10 is abundantly expressed in synovial fluids of RA patients [63,64] and has been linked with the control of bone resorption through inhibition of osteoclastogenesis [65]. Moreover, IL-10 is an anti-inflammatory cytokine with well-established antinociceptive properties [66,67]. Therefore, we can hypothesize that the increase in the bioavailability of EETs in TMJ tissue, through blocking their hydrolytic degradation by sEH enzyme, promotes increased tissue concentration of

IL-10, proving to be an essential pathway to control the inflammatory process into the albumin-induced arthritis in TMJ. Curiously, it has been recently reported that oral treatment with sEH inhibitor was unable to augment the protein levels of IL-10 in the knee joint of CIA mice [60]. The divergency found might be interpreted by the difference in tissue biology, the model used, and treatment routes. Neuroimmune interactions are unique to each tissue type and may depend on the cell types located in the tissue. In addition, sensory circuits from the TMJ area are modulated by the trigeminal system, while the knee joint is through the spinal system, that have different functional properties and response to injury or disease [68]. Lastly, peripheral administration of the sEH inhibitor could interact with resident cells over the TMJ, apart from the cells inside the articular joint, resulting in different anti-inflammatory and analgesic actions.

The sEH expression is increased in several diseases [24,69,70] and appears to be a common marker of tissue inflammation. Recently, our research group showed that the sEH enzyme inhibition by TPPU decreases bone loss by modulating host inflammatory response in an inflammatory bone resorption experimental model, similar to what occurs in arthritis [24,71]. Moreover, we have recently shown that the systemic inhibition of the sEH enzyme reduced pain, edema, and clinical score in arthritis-induced mice [60]. Herein, we use the albumin-induced arthritis in the TMJ model due to its singularity in affecting the TMJ directly, and thereby, allowing investigate the neuroimmune interactions and the circuit of pain regulated by the trigeminal system, which is distinct from the spinal system [68]. Taken together, our current results corroborate with previous studies to suggest that sEH enzyme inhibition could be a new target and strategy to relieve the pathologic effects of arthritis pain and inflammation.

Resident immune cells, particularly macrophages, play a critical role in inflammation and repair [72]. M1 macrophages (“classically activated”) release proinflammatory cytokines, are key components of host defense [73], and promote hyperalgesia [74,75]. In contrast, M2 macrophages (“alternatively activated”) secrete anti-inflammatory cytokine, promote tissue repair and produce analgesia [72,74,75]. Therefore, we finally investigated whether the peripheral anti-hypernociceptive and anti-inflammatory effect of TPPU could be, at least in part, associated with macrophage polarization. Our present results demonstrated that although the peripheral pretreatment with TPPU did not change the amount of macrophages in the TMJ tissue, it did significantly decrease the protein expression of iNOS, which is a marker for M1 macrophage, and did not alter the expression of MCR1, a marker for M2 macrophage. iNOS is closely associated with inflammation and involved in host defense and tissue damage. Particularly, iNOS is just expressed in activated cells, including macrophages [76,77]. It has been demonstrated that increased expression of NO leads to NF-kB signaling activation, and thereby, TNF-α, IFN-γ, IL-1β, IL-6, and other inflammatory cytokines production [76,78]. Previously, it has been demonstrated that the EETs can reduce inflammation thought the down-regulation of iNOS [79]. Thus, we can suggest that the increased peripheral levels of EETs, besides reducing arthritis-induced hypernociception and inflammation in the TMJ, also promotes an immunomodulatory activity by down-modulating the M1 macrophage.

In summary, our findings provided evidence that peripheral administration of the sEH enzyme inhibitor TPPU into the TMJ reduced the hypernociception and inflammation of albumin-induced arthritis in the rats' TMJ. Therefore, sEH enzyme inhibitors as TPPU could be considered as a new therapeutic approach in the treatment of inflammatory pain in the arthropathies.

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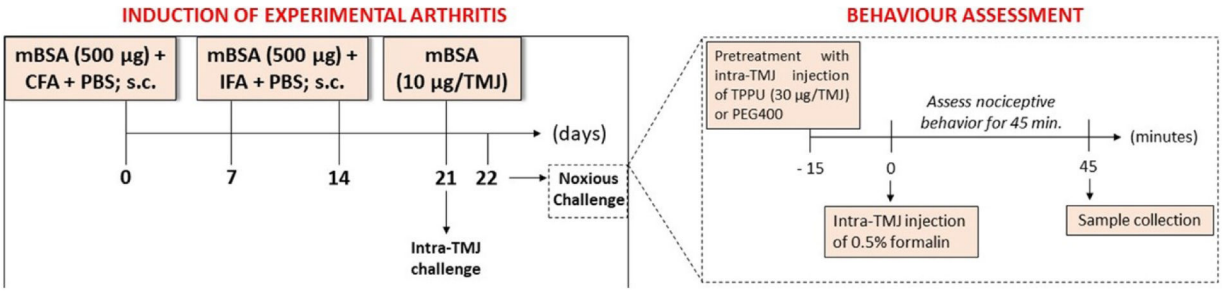

Fig. 1.

Experimental design of albumin-induced arthritis TMJ inflammatory hypernociception.

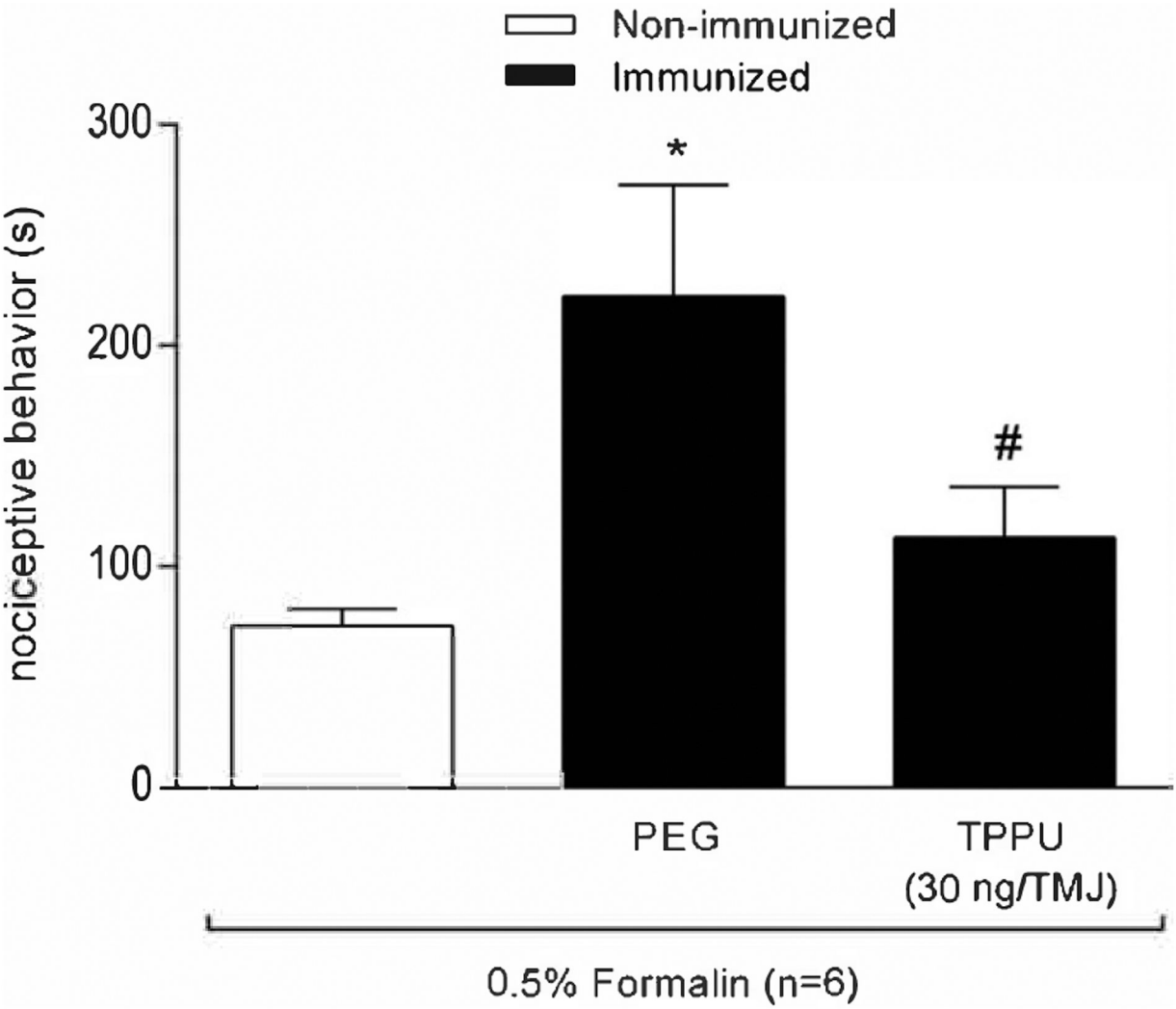

Fig. 2.

Effect of local pretreatment with TPPU on arthritis-induced TMJ hypernociception. The pretreatment with TPPU (30 ng/TMJ) reduced the arthritis-induced TMJ hypernociception in immunized rats ($p < 0.05$, Tukey test), as indicated by the symbol "#". The symbol "*" indicates a hypernociceptive response significantly greater than that induced in non-immunized rats (control group, $p < 0.05$, Tukey test). PEG: Polyethylene glycol, PEG400. Results are expressed as the mean ± SD of 6 animals per group.

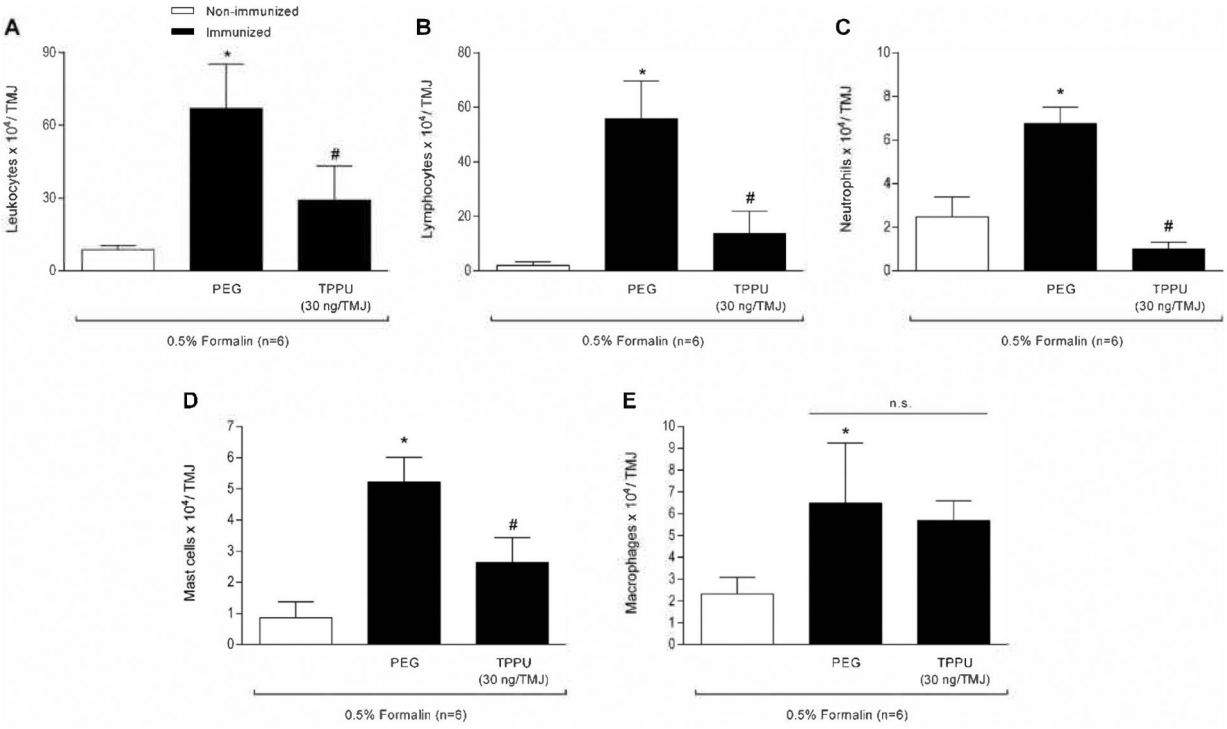

Fig. 3.

Effect of local pretreatment with TPPU on arthritis-induced inflammatory cells influx in the TMJ. The pretreatment with TPPU (30 ng/TMJ) reduced the arthritis-induced total leukocytes (A), lymphocytes (B), neutrophils (C), and mast cells (D) migration in the TMJ in immunized rats ($p < 0.05$, Tukey test), as indicated by the symbol "#". (E) The pretreatment with TPPU (30 ng/TMJ) did not affect the macrophage migration in the TMJ in immunized rats ($p > 0.05$, Tukey test). n.s.: non-significant. The symbol "*" indicates an inflammatory cell migration significantly greater than that induced in non-immunized rats (control group, $p < 0.05$, Tukey test). Results are expressed as the mean ± SD of 6 animals per group.

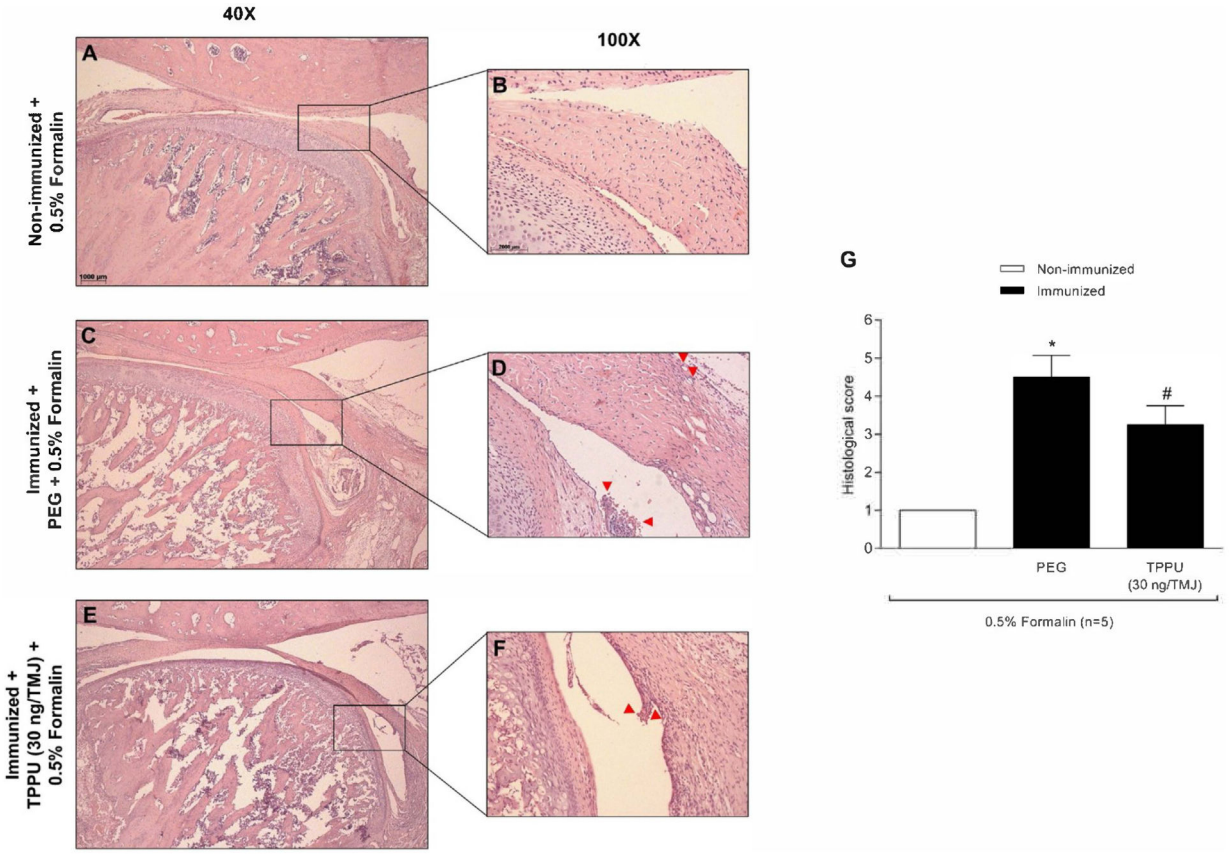

Fig. 4.

TPPU reduced the inflammatory cells influx on arthritis-induced in the TMJ. Histological score. Histological analysis and score were performed in TMJ sections stained with H&E (Hematoxylin and eosin stained, 40 and l00x magnification). Morphological features, showing the inflammatory infiltrate around the bone defects. In the non-immunized + 0.5% formalin control group (A and B), note few immune cells within stroma. The immunized + PEG + 0.5% formalin group demonstrated exacerbated leukocytes exudate (redhead arrow, D). TPPU-treated group note some immune cells within the stroma (E and F). A-F: Representative histological sections of experimental groups. (G) The pretreatment with TPPU (30 ng/TMJ) reduced the arthritis-induced inflammatory *pannus* in the TMJ of immunized rats ($p < 0.05$, Tukey test), as indicated by the symbol "#". The symbol "*" indicates inflammatory *pannus* significantly greater than that induced in non-immunized rats (control group, $p < 0.05$, Tukey test). The parameter analyzed was leukocyte infiltration. Results are expressed as the mean ± SD of 5 animals per group.

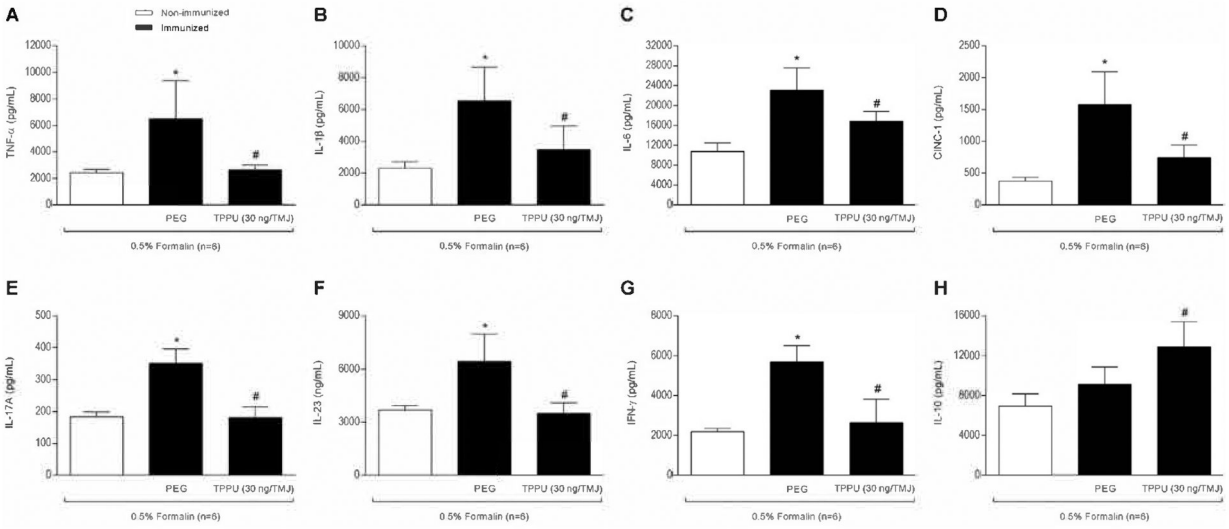

Fig. 5.

Effect of local pretreatment with TPPU on arthritis-induced release of inflammatory cytokines in the TMJ tissue. The pretreatment with TPPU (30 ng/TMJ) reduced the arthritis-induced increase of concentration of the proinflammatory cytokines TNF-α (A), IL-1β (B), IL-6 (C), CINC-1 (D), IL-17 (E), IL-23 (F), and IFN-γ (G) in the TMJ of immunized rats ($p < 0.05$, Tukey test), as indicated by the symbol "#". The symbol "*" indicates a concentration of proinflammatory cytokines significantly greater than that induced in non-inununized rats (control group, $p < 0.05$, Tukey test). (H) Pretreatment with TPPU (30 ng/TMJ) significantly increased the concentration of the anti-inflammatory cytokine IL-10 ($p < 0.05$, Tukey test), as indicated by the symbol "#". Results are expressed as the mean ± SD of 6 animals per group.

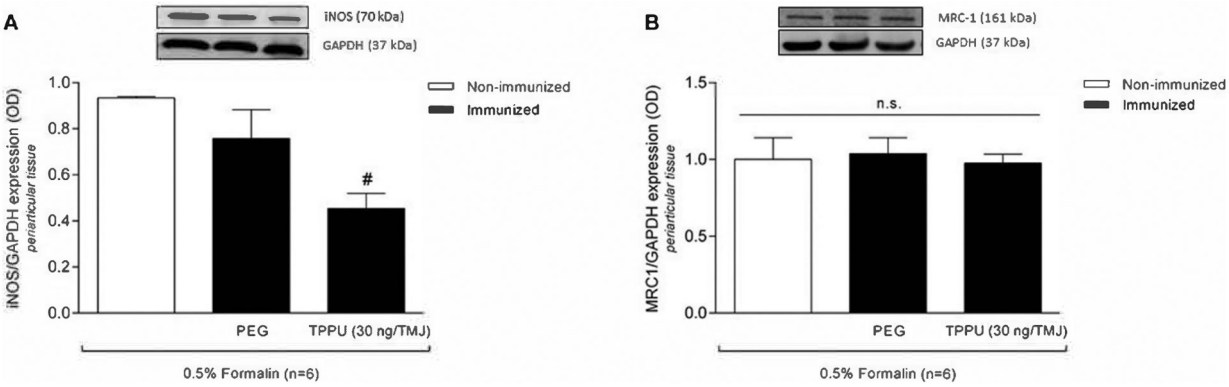

Fig. 6.

Effect of local pretreatment with TPPU on the expression of inducible nitric oxide synthase (iNOS) and C-type mannose receptor 1 (MCR1) in the TMJ tissue. (A) The pretreatment with TPPU (30 ng/TMJ) significantly decreased the expression of iNOS in the TMJ tissue of immunized rats ($p < 0.05$, Tukey test), as indicated by the symbol "#". (B) There is no difference among groups in the expression of MRC1 ($p > 0.05$, Tukey test). Representative iNOS, MCR1, and GAPDH bands of each group are displayed above the graph. n.s.: non-significant. Results are expressed as the mean ± SD of 6 animals per group.