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Article

Oral administration of eucalyptol reduces cell migration and pain-like behavior in zymosan-induced arthritis mice

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Rheumatoid arthritis (RA) is an inflammatory disease that utilizes nonbiologic and biologic drugs for appropriate disease management. However, high cost, adverse effects, reduced effectiveness, and risk of infection have stimulated the search for safer and more efficacious therapeutic strategies. In the present study, we aimed to evaluate the anti-inflammatory and analgesic properties of eucalyptol in an experimental model of arthritis. Mice were administered zymosan or saline intra-articularly. One hour before the zymosan administration, the mice were treated with oral eucalyptol (200-400 mg/kg) and vehicle. Cell influx, neutrophils, lymphocytes, and monocytes were measured in joint exudates. Joint pain was assessed using paw-pressure tests. Orally administered eucalyptol (200 and 400 mg/kg) significantly reduced cell influx, as well as neutrophils, lymphocytes, and monocytes, when compared with the control. Eucalyptol at a dose of 400 mg/kg significantly reversed joint pain and demonstrated analgesic activity (60%); however, 200 mg/kg failed to alter joint pain. These results indicate that oral eucalyptol promotes anti-inflammatory and analgesic activity in mice subjected to zymosan-induced arthritis.

Keywords: Eucalyptol. Anti-inflammatory. Analgesic. Arthritis. Zymosan.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovitis, joint pain, cartilage damage, and physical disabilities. Current medical management includes the use of nonbiologic disease-modifying antirheumatic drugs, corticosteroids, non-steroidal anti-inflammatory, and biologics (Mian, Ibrahim, Scott, 2019). Although treatment guidelines have recommended these therapies, factors such as high cost, adverse effects, reduced effectiveness, and risk of infection have invigorated the search for safer and more efficacious therapies (Aletaha, Smolen, 2018).

Eucalyptol (Euca) has been identified and extracted from aromatic plants and has demonstrated numerous applications during *in vivo* and *in vitro* studies, including anti-inflammatory and analgesic properties (Martins *et al.,* 2017; Caceres *et al.,* 2017). More recently, the effects of a Euca rich extract have been investigated in chronic diseases (Seol, Kim, 2016). In this context, RA is a common chronic disease associated with inflammatory and pain responses (Mian, Ibrahim, Scott, 2019; Aletaha, Smolen, 2018). An experimental study evaluating *Ocimum americanum L,* a plant species rich in Euca, has reported anti-inflammatory activity in an arthritis model (Yamada *et al.,* 2013). Although there exists evidence regarding the anti-inflammatory effects in arthritis models, few studies have explored the effects of Euca in this group of diseases. For example, a recent study reported that Euca alleviates pain and inflammation in a mouse model of gouty arthritis (Yin *et al.,* 2020). Thus, these observations suggest that Euca may have beneficial properties for managing other types of arthritis.

Therefore, in the present study, we aimed to evaluate the anti-inflammatory and analgesic properties of Euca in experimental zymosan (Zy)-arthritis in mice.

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MATERIAL AND METHODS

Chemicals

Zy and Euca were purchased from Sigma-Aldrich, São Paulo, Brazil.

Animals

In the present study, we used male Swiss mice weighing (20-25 g). The animals were obtained from the Central Animal Facility of the Faculty of Medicine, The Federal University of Ceará, Brazil. All efforts were made to minimize suffering. In total, 48 mice were procured and housed in temperature-controlled rooms with 12 h light/dark cycles and free access to water and food.

Animals were carefully monitored and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council (CMV), Guidelines for the Use of Animals in Research of the International Association for the Study of Pain (IASP), and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication). The Ethics Committee of The Federal University of Ceará approved the protocols employed (Reg. Nº. 13307).

Model of arthritis induced by Zymosan

Mice received an intra-articular (i.a.) injection of 0.1 mg Zy (Sigma, St. Louis, MO) (dissolved in sterile saline to 0.025 mL total volume) into the right knee joints. Control groups received i.a saline (Pinto *et al.*, 2013). One hour prior to the Zy injection, the mice were pretreated with Euca (200 and 400 mg/kg) and vehicle (2% Tween 80 in 0.9% NaCl) by oral gavage (peroral [p.o.]), as previously described (Lima *et al.,* 2013). Each group consisted of six mice.

Assessment of cell influx in joint aspirates

The synovial cavity of knee joints was washed with 0.05 mL saline containing 10 mmol/L EDTA (de Melo Nunes *et al.,* 2015). Synovial fluid was collected by aspiration, and total cell counts were assessed using a Neubauer chamber. Differential cell counts were evaluated using the panoptic Instant Prov™ staining kit (New ProvBrasil™).

Measurement of pain behavior

An experimenter blinded to group allocation assessed nociceptive behavior (regarded as joint pain) using an electronic pressure-meter nociception paw test. This method captures pain from the distally inflamed joint, possibly reflecting central sensitization (de Melo Nunes *et al.*, 2020). Animals were placed in acrylic cages (12 ×10 × 17 cm high) on a wire grid floor 15 min before testing in a quiet room. Stimulations were performed when animals were calm, without demonstrating exploratory behavior, urination, defecation, or resting of forepaws against walls. The electronic pressure-meter consists of a hand-held force transducer fitted with a polypropylene tip (Electronic von Frey aesthesiometer, Insight Equipamentos Científicos Ltda., Brasil). The polypropylene tip is applied perpendicularly to one of the five distal footpads of the right hind paw. The intensity of the stimulus is automatically recorded when the paw is withdrawn. The test was repeated three times until a difference of <1 g between measurements was obtained.

Statistical Analysis

All data were analyzed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). Results are presented as means ± standard deviation (SD). Differences between means were compared using one-way ANOVA, followed by Tukey's test. $P < 0.05$ was considered as significant.

RESULTS AND DISCUSSION

Anti-inflammatory activity

As shown in Figure 1A, Zy induced ($p<0.05$) leukocyte migration in the knee joint after 6 h; pretreatment with Euca (200 and 400 mg/kg, p.o.) markedly reduced ($p<0.05$) the Zy-induced cell influx in the joint exudate. We then analyzed cells based on their morphology and observed that Zy profoundly

induced ($p<0.05$) neutrophil, lymphocyte, and monocyte migration in the knee joint, as shown in Figure 1 (C-D). In contrast, pretreatment with Euca (200 and 400 mg/kg p.o.) inhibited the influx of neutrophils, lymphocytes, and monocyte into knee joints of mice presenting Zy-induced arthritis ($p<0.05$, Figure 1B-D).

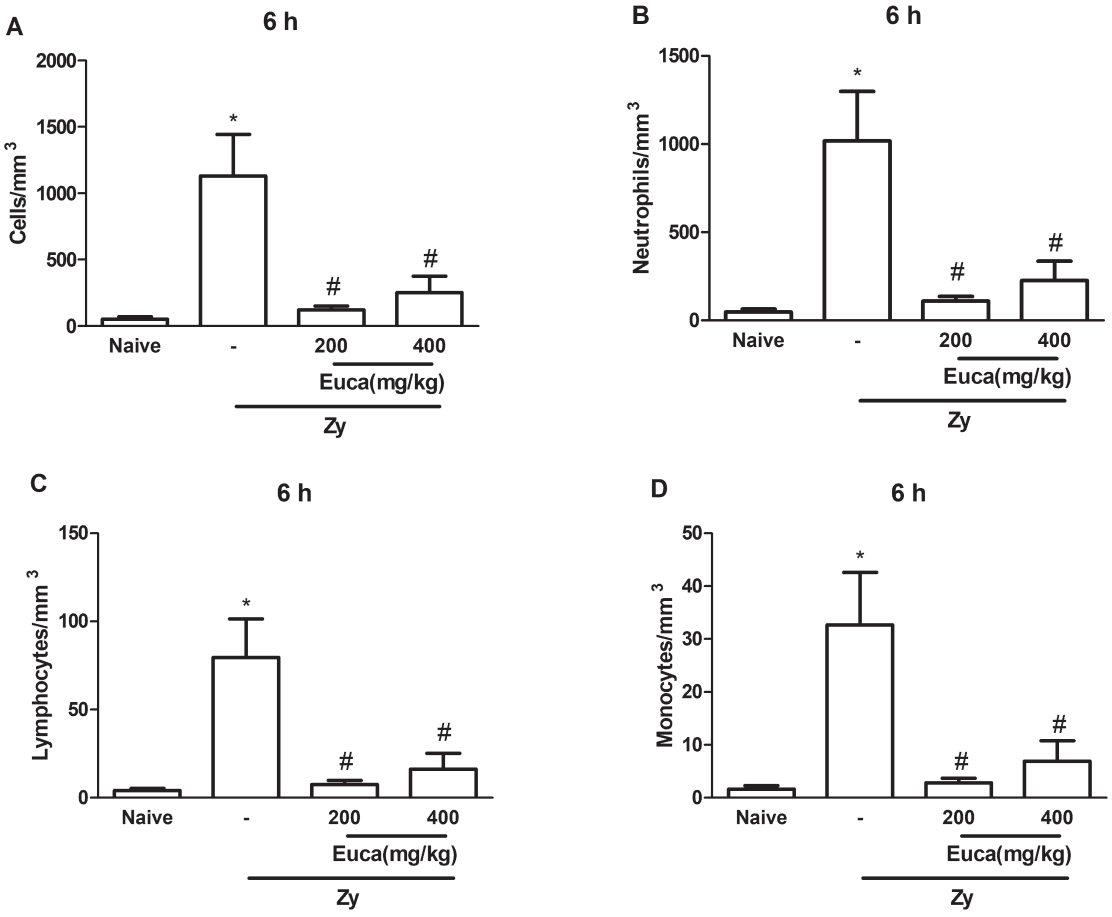

FIGURE 1 - Oral administration of eucalyptol (Euca) abrogates leukocyte migration promoted by zymosan-induced arthritis. (A) Cell influx, (B) neutrophils influx, (C) lymphocyte influx, and (D) monocyte influx were evaluated 6 h after zymosan (0.1 mg) administration. Euca (200-400 mg/kg) was administrated orally 1 h prior to zymosan. Data are mean ± standard deviation (SD) (n=6/group). *$p<0.05$ *vs*. naive and #$p<0.05$ *vs*. zymosan (-) using one-way ANOVA followed by Tukey's test.

Next, we performed *in vivo* investigations to assess the effect of Euca on inflammation. Euca (200 and 400 mg/kg), administered orally or intraperitoneally, reportedly decreases the levels of myeloperoxidase (neutrophil marker) and inflammatory cell infiltration in mice tissues (Lima *et al.,* 2013; Kim, Lee, Seol, 2015). However, the appropriate dose varies based on the disease model employed. For instance, a high dose of Euca (800 mg/kg) injected for 3 days effectively reduced thioacetamide-induced hepatoxicity and/or immunotoxicity (Kim *et al.,* 2004). In addition, the anti-inflammatory activity of Euca (300 mg/kg) was revealed in a gout model, with inhibition of inflammatory cell infiltration in the ankle joint (Santos *et al.,* 2004; Yin *et al.,* 2020).

Our findings corroborated with previous evidence and revealed that pretreatment with Euca reduced neutrophil, lymphocyte, and monocyte infiltration in Zy-induced arthritis. These cells have been shown to play an important role in the pathogenesis of arthritis. Neutrophils generate peroxynitrite, which in turn increases cell influx, myeloperoxidase activity, and nitric oxide into the knee joint during Zy-induced arthritis (Bezerra *et al.,* 2007). Lymphocytes directly modulate interferon, interleukin (IL)-10, and nitric oxide release (Kolaczkowska *et*

al., 2008). During synovial inflammation, monocytes stimulate the synthesis of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-1β, and IL-6 (Rana *et al.,* 2018). Notably, all these mediators reportedly promote inflammation in Zy-induced arthritis models (Pinto *et al.,* 2013). However, additional investigations are needed to determine whether Euca can modulate the release of these cytokines during Zy-induced arthritis.

Analgesic Activity

Next, we evaluated whether Euca could attenuate pain promoted by Zy-induced arthritis using the electronic pressure-meter nociception paw test. We observed that Zy diminished the mechanical paw withdrawal threshold at 3 h and 5 h when compared with the naive group ($p<0.05$, Figure 2). Mice pretreated with Euca (400 mg/kg p.o.) exhibited a higher paw withdrawal threshold at 3 h and 5 h after Zy injection than the non-pretreated mice presenting Zy-induced arthritis. Notably, pretreatment with Euca (200 mg/kg p.o.) did not reduce joint pain.

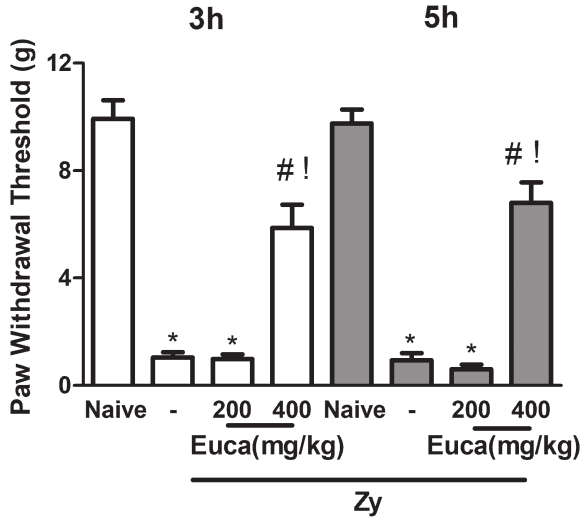

FIGURE 2 - Oral administration of eucalyptol (Euca) ameliorates pain-like behavior in zymosan-induced arthritis in a dose-dependent manner. Paw withdrawal threshold was evaluated using the electronic pressure-meter nociception paw test at 3 h and 5 h after zymosan (0.1 mg) administration. Euca (200-400 mg/kg) was administrated orally 1 h prior to zymosan. Open and closed bars represent values at 3 h and 5 h, respectively. Data are mean ± ± standard deviation (SD) (n=6/group). *$p<0.05$ *vs.* naive, #$p<0.05$ *vs.* zymosan (-) and ! $p<0.05$ *vs.* Euca (200 mg/kg) using one-way ANOVA followed by Tukey's test.

The antinociceptive effect of Euca has been previously reported in a pain model (Santos, Rao, 2000). In a model of gouty arthritis, analgesic activity has been associated with the downregulation of cell migration in joint tissues (Yin *et al.,* 2020). Similar to the gout model, in the Zy-induced arthritis model, migration of inflammatory cells to the joint space results in the release of inflammatory mediators, causing pain-like behavior during Zy-induced arthritis (Bezerra *et al.,* 2007; Pinto *et al.,* 2013; de Melo Nunes *et al.,* 2015). Therefore, our results indicated that Euca inhibited the pain-like behavior in a Zy-induced mouse model by decreasing cell migration.

Collectively, our results suggest that oral Euca has anti-inflammatory and analgesic activity in the Zy-induced arthritis mouse model. This study could provide an experimental basis for elucidating the mechanism of Euca, as well as for developing therapeutic strategies for employing Euca in RA management.

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