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# Citronellol, a natural acyclic monoterpene, attenuates mechanical hyperalgesia response in mice: Evidence of the spinal cord lamina I inhibition

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ABSTRACT

We evaluated the anti-hyperalgesic effect of citronellol (CT) and investigated the spinal cord lamina I involvement in this effect. Male mice were pre-treated with CT (25, 50 and 100 mg/kg, i.p.), indomethacin (10 mg/kg, i.p.), dipyrone (60 mg/kg, i.p.) or vehicle (saline + Tween 80 0.2%). Thirty minutes after the treatment, 20 μL of carrageenan (CG; 300 μg/paw), $PGE_2$ (100 ng/paw), dopamine (DA; 30 μg/paw) or TNF-α (100 pg/paw) were injected into the hind paw subplantar region and the mechanical threshold was evaluated with an electronic anesthesiometer. The CT effect on edema formation was evaluated after the right paw subplantar injection of CG (40 μL; 1%) through the plethysmometer apparatus. To evaluate the CT action on the spinal cord, the animals were treated with CT (100 mg/kg; i.p.) or vehicle (Saline + Tween 80 0.2%; i.p.) and, after 30 min, 20 μL of CG (300 μg/paw; i.pl.) was injected. Ninety minutes after the treatment, the animals were perfused, the lumbar spinal cord collected, crioprotected, cut and submitted in an immunofluorescence protocol for Fos protein. CT administration produced a significantly reduction ($p < 0.05$) in the mechanical hyperalgesia induced by CG, TNF-α, $PGE_2$ and DA when compared with control group. The treatment with CT also significantly ($p < 0.05$) decreased the paw edema. The immunofluorescence showed that the CT decrease significantly ($p < 0.05$) the spinal cord lamina I activation. Thus, our results provide that CT attenuates the hyperalgesia, at least in part, through the spinal cord lamina I inhibition.



## 1. Introduction

Pain perception is a defense system that alarms the organism about danger and produces immediate response to stress factors. In inflamed tissue, however, primary sensory neurons become hypersensitive, produce pain response to normally innocuous stimulation or enhanced pain response to painful stimulation [1]. This response is established with the participation of several mediators, such as neurotrophic factors, neuropeptides, prostanoids, kinins and some cytokines [2,3].

The carrageenan (CG), a high-value seaweed hydrocolloid, have been used in the inflammatory pain studies, once it induces mechanical hyperalgesia through a cascade of cytokines, like TNF-α, IL-6 and IL-1β, released by resident or migrating cells initiated by production of bradykinin, with consequent synthesis of prostaglandins and release of sympathetic amines [4,5].

The Nonsteroidal Anti-inflammatory Drugs (NSAIDs) is a name for a number of chemically distinct drugs, representing the most widely used drug class. The NSAIDs, such indomethacin and

*Abbreviations:* NSAIDs, Nonsteroidal Anti-inflammatory Drugs; CT, citronellol; CG, carrageenan; TNF-α, tumor necrosis factor-alpha; $PGE_2$, prostaglandins-$E_2$; DA, dopamine; BSA, bovine serum albumin; PBS, phosphate buffer (0.01 M) saline isotonic; COX, cyclooxygenase; TNFR1, tumor necrosis factor receptor 1; TNFR2, tumor necrosis factor receptor 2; IL, interleukin; KC, keratinocyte-derived chemokine; $EP_2$, prostaglandin $E_2$ receptor; PAG, periaqueductal gray area; RVM, rostral ventromedial medulla; CNS, central nervous system.

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dipyrone, inhibitors of prostaglandins synthesis, are used for the pain inhibition at the site of inflammation. However, significant side effects and low efficacy of NSAIDs in quite a number of inflammatory states primed search of other targets for inflammatory pain relief [1,6,7].

For this reason, there are many studies focusing in the search of new therapeutic options for treating painful conditions. In this context is inserted the monoterpenes, a compound group formed from the coupling of two isoprene units (C10), constituting 90% of the essential oils. Some systematic reviews described the analgesic and anti-inflammatory activities of some monoterpenes, demonstrating the therapeutic potential of these compounds, including to development of pharmaceutical products for pain [8–11].

Citronellol (CT) is a monoterpene compound prevalent in essential oils of various aromatic plant species, such as *Cymbopogon citrates* and *Cymbopogon winterianus* [12,13], and its hypotensive, vasorelaxant, anticonvulsant, analgesic and anti-inflammatory profiles are described in the literature [14–17]. However, there are few data that have evaluated the effects of CT on inflammatory pain. Hence, the purpose of the present study was to evaluate the anti-hyperalgesic effect of CT in mice and investigate the involvement of the spinal cord lamina I in this possible effect.

## 2. Materials and methods

### 2.1. Chemicals

Carrageenan (CG), tumor necrosis factor-alpha (TNF-α), prostaglandins-$E_2$ ($PGE_2$), dopamine (DA), Tween 80, ((S)-(−)-B-citronellol, CT, 97% purity), glycerol solution, glycine and bovine serum albumin (BSA) were purchased from Sigma (USA). Indomethacin and dipyrone were obtained from União Química (Brazil). Rabbit anti-Fos k-25 was obtained from Santa Cruz Biotechnology (USA) and the donkey anti-rabbit Alexa Fluor 488 was purchased from Life Technologies (USA).

### 2.2. Animals

Adult (3-month-old) male albino Swiss mice (28–32 g) were randomly housed in appropriate cages at 21 ± 2 °C with a 12-h light: dark cycle (light from 06:00 to 18:00), with free access to food (Purina®, Brazil) and tap water. All experiments were carried out between 09:00 am and 02:00 pm in a quiet room. Experimental protocols were approved by the Animal Care and Use Committee at the Federal University of Sergipe (CEPA/UFS #72/11). All tests were carried out by the same visual observer, double-blinded and all efforts were made to minimize the number of animals used as well as minimize any discomfort.

### 2.3. Hyperalgesia induced by CG, TNF-α, $PGE_2$ and DA

The hyperalgesia protocols was performed as previous described [18,5,19]. The animals were divided into five groups ($n = 6$, per group) and treated with vehicle (Saline + Tween 80 0.2%, i.p.), CT (25, 50 or 100 mg/kg, i.p.), indomethacin (10 mg/kg, i.p.) or dipyrone (60 mg/kg, i.p.). Thirty minutes after the treatment, 20 μL of CG (300 μg/paw), $PGE_2$ (100 ng/paw), DA (30 μg/paw) or TNF-α (100 pg/paw) were injected subcutaneously into the subplantar region of the hind paw. The mechanical hyperalgesia was evaluated at 0.5, 1, 2 and 3 h after the hyperalgesic agents injections.

### 2.4. Mechanical hyperalgesia measurement

Mechanical hyperalgesia was tested in mice as previous reported [18]. In a quiet room, the mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors for 15–30 min. before the test. This method consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic anesthesiometer; Insight®, São Paulo, Brazil) adapted with a polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the withdrawal of the paw followed by clear flinching movements. After this response, the pressure intensity was automatically recorded. The intensity of stimulus was obtained by averaging five measurements taken with minimal intervals of 3 min. The animals were tested before and after the treatment.

### 2.5. Paw edema measurement

The CT effect on edema formation caused by the intraplantar injection of CG was analyzed according to the method previously reported [20]. The animals were divided in five groups ($n = 6$, per group) and treated with vehicle (Saline + Tween 80 0.2%, i.p.), CT (25, 50 or 100 mg/kg, i.p.), or indomethacin (10 mg/kg; i.p.). The right paw volume was measured by the water column dislocation of a plethysmometer (Insight®, Brazil) before (time zero) and 1, 2, 3, 4, 5 and 6 h after subplantar injection of 40 μL of CG (1%). The paw edema was expressed (in milliliter) as the difference between the volume of the paw after and before CG injection. The area under the curve (AUC [0–240 min]; in milliliter per minute) was also calculated using the trapezoidal rule.

### 2.6. Immunofluorescence

To evaluate the action of the test drug on the spinal cord, the animals ($n = 6$, per group) were treated with CT (100 mg/kg; i.p.) or vehicle (Saline + Tween 80 0.2%; i.p.) and, after 30 min, 20 μL of CG (300 μg/paw) was injected subcutaneously into the subplantar region of the hind paw. One group did not receive any kind of treatment (sham group), which was used as a baseline control. Ninety minutes after the treatment, all animals were perfused and the lumbar spinal cord collected and crio-protected for immunofluorescence processing to Fos protein. The time for realization of immunofluorescence protocol was based on the studies of Barr [21] and Bai et al. [22].

Frozen serial transverse sections (20 μm) of all spinal cords were collected on gelatinized glass slides. The tissue sections were stored at −80 °C until use. The sections were washed with phosphate buffer (0.01 M) saline isotonic (PBS) 5 times for 5 min. and incubated with 0.01 M glycine in PBS for 10 min. Non-specific protein binding was blocked by incubation of the sections for 30 min. in a solution containing 2% BSA. Then, the sections were incubated overnight with rabbit anti-Fos as primary antibodies (1:2000). Afterwards, the sections were incubated for two hours with donkey anti-rabbit Alexa Fluor 594 as secondary antibodies (1:2000). The cover slip was mounted with glycerol solution. As an immunofluorescence control for non-specific labeling, sections were incubated without primary antibody. After each stage, slides were washed with PBS 5 times for 5 min.

### 2.7. Acquisition and analyses of images

A blinded investigator took 20 pictures from Fos positive lumbar spinal cords areas for each animal using an Olympus IX2-ICB (Tokyo, Japan). The lumbar spinal cord regions were classified according to Paxinus and Franklin Atlas [23]. After that, the same