Protective effect of *Chresta martii* extract on the zymosan-induced temporomandibular joint arthritis in rats

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ABSTRACT

Objective: *Chresta martii* is broadly used by folk medicine due to its anti-inflammatory effects, but there is a lack of preclinical data on its pharmacological mechanisms. This study investigated the efficacy of *Chresta martii* ethanolic extract (CEE) in the zymosan-induced temporomandibular joint arthritis (TMJ) and evaluated the possible role of TNF- α , nitric oxide (NO), and heme oxygenase-1 (HO-1).

Methods: Male Wistar rats (160–220 g) were pre-treated with CEE (100, 200 or 400 mg/kg; v.o) 1 h before zymosan injection (2 mg; i.art). Mechanical hypernociception (g) was assessed 4 h later. The trigeminal ganglion was collected for TNF- α quantification (ELISA), total cell count and myeloperoxidase activity (MPO) were assayed in the synovial lavage 6 h after arthritis induction. Additionally, animals were pre-treated with L-NAME (30 mg/kg; i.p.) or ZnPP-IX (3 mg/kg, s.c.) to assess the involvement of NO and HO-1, respectively.

Results: CEE 400 mg/kg (v.o) increased ($p < 0.05$) hypernociception threshold, reduced the cell counts and MPO activity in the synovial lavage, as well as decreased TNF- α levels in the trigeminal ganglion. ZnPP-IX abolished the analgesic effect of CEE, but not L-NAME.

Conclusion: The anti-inflammatory and antinociceptive effects of CEE depended on the HO-1 pathway integrity and TNF- α suppression.

1. Introduction

Temporomandibular joint (TMJ) disorders encompass clinical conditions that culminate in TMJ pain, impairing quality of life.¹ Animal models that allow the study of the mechanisms involved in such scenario contributes to understanding the physiopathology of TMJ disorders. Once our major interest is in unraveling the underlying TMJ pain mechanisms, we developed an experimental model of TMJ inflammatory hypernociception.^{2,3}

During hypernociception, NO activates soluble guanylate cyclase (cGC), increasing intracellular levels of cyclic guanosine

monophosphate (cGMP), which activates potassium channels.⁴ These channels induce membrane hyperpolarization, thus, reducing depolarization and the firing of action potentials, culminating in analgesia.⁵ While HO-1 is induced in inflammatory cells by heme, endotoxins, cytokines, nitric oxide, and other mediators produced during inflammatory responses.^{6–8} HO-1 induction provides negative feedback for cellular activation and the synthesis of pro-inflammatory mediators.^{9–11} Inflammatory conditions induce HO-1 and the expression of this protein is an adaptive mechanism that dampens inflammatory insults.¹²

Developing novel therapeutics to tackle inflammatory pain involves

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the search for biologically active molecules, propelling scientific studies further into the identification of ideal candidates for this task. Additionally, this search is justified in face of the concerning side-effects of many anti-inflammatory archetypes such as non-steroidal anti-inflammatory drugs or glucocorticoids, which in turn impose a limitation to long-term treatment. In this regard, there has been a growing interest in alternative therapies and the use of natural products derived from algae or plants in recent years has called much attention upon such hot topic.^{13–15}

The *Chresta* genus belongs to the family Asteraceae¹⁶ consists of twelve species (*Chresta amplexifolia*, *Chresta harleyi*, *Chresta martii*, *Chresta pinnatifida*, *Chresta curumbensis*, *Chresta speciosa*, *Chresta scapigera*, *Chresta angustifolia*, *Chresta plantaginifolia*, *Chresta sphaerocephala*, *Chresta exsucca*, *Chresta pycnocephala*) which are broadly spread across the North and Northeast in Brazil.¹⁷ Among the mentioned species, *Chresta martii* is reportedly used by folk medicine to exert anti-inflammatory effects and preclinical studies have confirmed these claims.^{18–22}

Thus, once the anti-inflammatory and antinociceptive potentials of *C. martii* have been underexplored so far, here we tested whether its ethanolic extract orally administered would reduce inflammatory and nociceptive parameters in a preclinical model of TMJ pain in rats and the possible involvement of NO and HO-1 pathway. Thus, we produce the first evidence ever given that support its anti-inflammatory and analgesic potential beyond the gastroprotective effects.

2. Material and methods

2.1. Animals

Seventy-eight male Wistar rats (160–220 g) were housed in plastic cages (n = 6) with food and water *ad libitum* in a temperature-controlled room (23 ± 2 °C), at a 12/12-hour light-dark cycle. All experiments were designed to minimise animal suffering and to use the minimum number of animals required to achieve a valid statistical evaluation. These experimental protocols were conducted following the Institutional Animal Care and had the approval of the local ethics committee under the registration number CEUA 57/10 of the Federal University of Ceará - campus Sobral (2014–2016), where the study was carried out.

2.2. Botanic material

C. martii flowers and leaves were collected at the following coordinates: longitude –37.940, latitude –9.5563 to –9.5548, altitude 130 m. An exsiccate (14602) was registered in the local herbarium. The dry powder of flowers and leaves was produced using an ethanolic solution (50%) at room temperature (28 ± 3 °C) for 48 h. After filtration, the dark green solution was concentrated at 50 °C under negative pressure until dried and stored (–20°C). The dried extract was resuspended in 0.9% saline solution on the experimental day and was orally administered at 100, 200, 400 mg/kg (v.o.)

2.3. Drug treatment

The rats were randomly allocated to different groups and orally received (v.o.) *C. martii* ethanolic extracts (CEE) at 100, 200, 400 mg/kg or 0.9% saline solution 1 h before zymosan challenge via intra-articular (i.art.) injection. A positive control group consisted of indomethacin-treated subcutaneously (s.c.) rats (5 mg/kg) 1 h before zymosan injection. The role of the HO-1 and NO pathways in the CEE effect was determined by pre-treating animals with ZnPP-IX (3 mg/kg, s.c.), a specific HO-1 inhibitor, alone or followed by administration of CEE (400 mg/kg; v.o.) 30 min later; or L-NAME via intraperitoneal injection (i.p.) (30 mg/kg), a non-selective NO inhibitor, alone or followed by CEE (400 mg/kg; v.o.) 60 min later. One hour after these

Fig. 1. (a) Effects of the ethanolic extract of *Chresta martii* (DC.) H. Hob on the mechanical threshold, the leukocyte infiltration and MPO activity in the synovial lavage in rats after the zymosan-injection into the TMJ. CEE (100, 200, or 400 mg/kg) was orally administered 1 h before zymosan challenge. Wistar rats received zymosan (2 mg/40 μL; left TMJ). Sham groups received 40 μL of a saline solution (i.art.). (a) The mechanical threshold was assessed at the fourth hour after intra-articular injections of zymosan or saline solution. (b, c) The leukocyte counting and MPO activity were determined 6 h after intra-articular injections of zymosan or saline solution. Data are represented as means ± S.E.M (n = 6). **p* < 0.05 in relation to the zymosan group; #*p* < 0.05 in relation to the sham group (ANOVA, followed by Bonferroni's test).

Fig. 2. Effects of ZnPP-IX (a selective inhibitor of heme oxygenase-1) and L-NAME (non-selective inhibitor of nitric oxide synthase) on the TMJ mechanical threshold and leukocyte infiltration after zymosan injection in the *Chresta martii*-treated animals.

Wistar rats were pre-treated with ZnPP-IX (3 mg/kg; s.c.) or L-NAME (30 mg/kg; i.p.) after 30 min and 60 min received orally the extract of *Chresta martii*, respectively for each group. After an hour, animals received an intra-articular injection of zymosan (2 mg; 40 µL) into the left TMJ. Sham groups receive saline solution injection (40 µL; i.art). (a, c) The mechanical threshold was assessed 4 h after zymosan injection. (b, d) Leukocyte infiltration was assessed four and 6 h after the zymosan injection. Data are represented as means $\pm$ S.E.M (n = 6). *p < 0.05 in relation to the zymosan group; #p < 0.05 in relation to the sham group; δp < 0.05 in relation to groups treated with *Chresta martii* extract and zymosan (ANOVA, followed by Bonferroni's test).

treatments, zymosan was injected into the left TMJ. Inflammatory hypernociception and total cell count were determined as previously described.²

2.4. TMJ hypernociception induction

Rats underwent 4% isoflurane anesthesia and zymosan (Sigma Aldrich) dissolved in sterile 0.9% saline solution (2 mg; 40 µL; i.art.) was injected into the left TMJ via a 30-gauge needle.² Control groups orally received saline solution before zymosan or saline solution injection into the TMJ, this latter group is also known as sham group. Mechanical hypernociception peaks 4 h and cell influx peaks 6 h after zymosan injection.² These time points were used to assess head withdrawal threshold, total cell counting, and myeloperoxidase (MPO) activity.

2.5. Head withdrawal threshold (g)

TMJ hypernociception was evaluated by determining the force intensity to cause head withdrawals. These measurements were

performed by a blind examiner to treatments who used a digital anesthesiometer (Insight, Ribeirão Preto, SP, Brazil), which consists of a rigid filament coupled to an electronic transducer that measures the response threshold (g) when the filament is applied to the TMJ.^{1,2} The facial areas to be tested were carefully shaved, and the animals were individually placed in plastic cages 45 min before behavioral assessment. The rats were subjected to conditioning sessions of head withdrawal threshold measurements in the testing room for 4 consecutive days. On the fifth day, the basal force threshold was recorded three times before and 4 h after the injections of either zymosan or vehicle (i.art.).

2.6. Total cell number and MPO activity in the synovial lavage

Rats were culled under general anesthesia and exsanguinated 6 h zymosan challenge. Then, superficial tissues were dissected and the TMJ cavity was exposed and washed twice with 50 µL of an EDTA solution (1.77 mg EDTA/1 mL phosphate-buffered solution) to collect the synovial fluid. Neubauer chamber was used to count the total numbers of cells in the synovial lavage, while the MPO activity was

Fig. 3. TNF- α levels in the trigeminal ganglion after zymosan injection in the *Chresta martii*-treated animals.

Wistar rats received orally the extract of *Chresta martii*. After an hour, animals received an intra-articular injection of zymosan (2 mg; 40 μ L) into the left TMJ. Sham groups receive saline solution injection (40 μ L; i.art). The TNF- α concentration in the trigeminal ganglion was determined 6 h after the zymosan injection. Data are represented as means $\pm$ S.E.M (n = 6). * p < 0.05 in relation to the zymosan group; # p < 0.05 in relation to the sham group (ANOVA, followed by Bonferroni's test).

determined as described.²³ MPO activity in the collected synovial lavage was assayed by measuring the change in absorbance at 450 nm using o-dianisidine dihydrochloride and 1% hydrogen peroxide. The results are reported as the MPO units/joint lavage. A unit of MPO activity was defined as the conversion of 1 μ mol of hydrogen peroxide to water in 1 min at 22 °C.

2.7. TNF- α levels

The trigeminal ganglia were harvested 6 h after the zymosan challenge, homogenized in RIPA Lysis Buffer System (Santa Cruz Biotechnology, USA), and centrifuged (10000 rpm, 15 min, 4 °C). The cytokine levels were quantified by TNF- α -Rat TNF- α /TNFSF1A Quantikine ELISA Kit (R&D Systems, catalog number RTA00). The absorbance was measured at 450 nm and TNF- α concentrations were expressed as pg/mL.

2.8. Statistical analysis

Data are presented as the means $\pm$ S.E.M. or medians. Differences between means were compared using one-way ANOVA followed by Bonferroni's test. A probability value of p < 0.05 indicated significant differences.

3. Results

CEE at 400 mg/kg significantly increased the head withdrawal threshold (p < 0.05) (Fig. 1a) and reduced the leukocyte influx into the TMJ 6 h after zymosan challenge (Fig. 1b) in comparison to the respective controls. The MPO activity decreased after CEE treatment at all tested doses (Fig. 1c), being the 400 mg/kg posology just as effective in reducing this parameter as indomethacin.

Regarding the involvement of the HO-1 pathway, ZnPP-IX abolished the analgesic effects of CEE, but no significant difference was found regarding the leukocyte infiltration (Fig. 2a and b). Concerning the NO pathway, Fig. 2c and d depict no change in the mechanical threshold

nor leukocyte migration upon NOS inhibition by L-NAME, suggesting that the pharmacological effects of CEE do not depend upon the NO pathway integrity.

Additionally, the CEE treatment reduced the TNF- α levels in the trigeminal ganglion. This inflammatory cytokine plays a crucial role in nociception and accounts for hyperalgesia maintenance, probably acting as a neuromodulator in the trigeminal ganglion, facilitating pain (Fig. 3).

4. Discussion

Here, we investigated the CEE antinociceptive and anti-inflammatory effects on an experimental model of zymosan-induced TMJ inflammatory pain. CEE at 400 mg/kg increased head withdrawal thresholds, reduced cellular influx into the TMJ, MPO activity in the synovial lavage, and TNF- α levels in the trigeminal ganglion. Its analgesic effects only occurred in the presence of L-NAME, but not when ZnPP-IX was administered.

The screening for novel therapeutics contributes to the study of inflammatory diseases.^{24,25} This study evaluated the biological effects of *C. martii*, which is reportedly used to treat inflammatory conditions.^{18–22} Our group has been investigating the pharmacological properties of this species since 2012 and its mechanisms of action have been gradually elucidated.^{26,27} Given the above, we decided to assess whether CEE would improve inflammatory pain.

The zymosan-induced arthritis model is a classical inflammatory response associated with spontaneous pain. It presents NO formation in TMJ exudates, an increase in iNOS expression in the articular disc, articular cartilage chondrocytes, and synovial cells.^{1,2} Cellular migration to tissues occurs via a cascade of events.^{28–31} Our results showed that CEE at 400 mg/kg reduced cellular migration of total leukocytes and MPO activity in the synovial lavage.

NO is part of a system of endogenous gaseous mediators produced by enzymes called nitric oxide synthases (NOS).³² Our results showed that CEE did not interfere in the NO pathway. These findings corroborate with previous studies that suggested no dependence on NOS for analgesic effects of some natural products.^{21,22}

Carbon monoxide (CO) is a product of the inducible (HO-1) and constitutive (HO-2) heme-oxygenases. CO activates soluble guanylate cyclase (sGC), producing cGMP. Inflammatory triggers can lead to CO production in several cell types and HO-1 expression reduces inflammation.^{10,33} Our group demonstrated that the HO-1/CO/Biliverdin pathway plays an anti-inflammatory and anti-nociceptive role in the TMJ arthritis,² acetic acid-induced writhes and carrageenan-induced paw edema.³⁴ Here, pre-treatment with ZnPP-IX prevented CEE effects, showing the dependence of CEE on the HO-1 pathway integrity.

Furthermore, CEE treatment reduced TNF- α levels in the trigeminal ganglion. The role of TNF- α in the physiopathology of temporomandibular joint arthritis has been demonstrated.⁴ TNF- α , as a neuromodulator, plays a crucial role in the pathophysiology of pain as an inflammatory trigger due to the zymosan stimulation of afferent terminal fibers of the trigeminal nerve.

Considering the given data, our study demonstrated the use of CEE in a context of TMJ inflammation, providing possible mechanisms of action. Additionally, given the well-known side-effects of conventional drugs used to treat TMJ inflammatory pain, these findings could point out possible alternatives with low risk of side-effects to treat such conditions. Considering all the data acquired so far regarding CEE safety and anti-inflammatory potential, small clinical trials using this ethanolic extract could be carried out in a small sample of individuals with clinically diagnosed inflammatory TMJ pain given especial attention to possible side-effects, outcomes, and true association between treatment and pain relieve. Obviously, this approach would have limitations due to small sample size, but it would be a better choice than employing this treatment to a larger sample.

5. Conclusion

Our results showed that CEE promoted antinociceptive and anti-inflammatory activities. Our findings suggest that CEE at 400 mg/kg can reduce nociceptive and inflammatory parameters upon HO-1 integrity and TNF- α suppression. Thus, given the limitations of an experimental and well-controlled animal study, our findings may support the popular use of CEE to treat inflammatory conditions.

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Declaration of competing interest

The authors declare no conflict of interest regarding the publication of this article.

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