Original Research
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Orofacial antinociceptive effects of perillyl alcohol associated with codeine and its possible modes of action

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Abstract: This study evaluated the orofacial antinociceptive effect of (S)-(-)-perillyl alcohol (PA) associated with codeine (C) and investigated the possible molecular anchorage mechanisms of PA. Mice (n = 5 per group) were treated with PA alone and associated with codeine and assigned to the following groups: 75.0 mg/kg PA; 75.0 mg/kg PA + C 30 mg/kg; PA 37.5 mg/kg + C 15.0 mg/kg; C 30.0 mg/kg; and control. Nociception was induced by formalin, capsaicin, and glutamate, and was quantified based on the duration (in seconds) of face grooming. The possible mechanisms of action were evaluated by molecular docking study. In the formalin test, PA75/C30 presented an effect in the neurogenic ($p < 0.0001$) and inflammatory ($p < 0.005$) phases. Mice treated with PA75 ($p < 0.0001$) and PA75/C30 ($p < 0.0005$) showed a reduced nociceptive behavior in the capsaicin test. Glutamate-induced nociception also was blocked by PA75 ($p < 0.0005$) and C30 ($p < 0.0005$). The molecular anchorage analysis indicated high negative binding energy values for the evaluated receptors, especially glutamate receptors (AMPA -79.57 Kcal/mol, mGLUR6 -71.25, and NMDA -66.33 Kcal/mol). PA associated with codeine showed orofacial antinociceptive activity, with theoretical evidence of interaction with glutamate receptors.

Keywords: Facial Pain; Analgesics, Opioid; Biological Products; Drug Synergism; Pain Management.

Introduction

Orofacial pain is commonly referred to as a morbid condition of the oral cavity or face. It can be associated, however, with head and neck pain, primary headache, cervicalgia, or rheumatic disorders (such as fibromyalgia and rheumatoid arthritis).[1-3] A prevalence of 51.5% for dental pain among 8 to 10 year-old Brazilian schoolchildren has been self-reported.[4] This indicates that dental pain is an important public health problem.[4]

The pharmacological treatment of orofacial pain can be made using opioid and non-opioid analgesics and anti-inflammatory drugs. Moreover, antidepressants and muscle relaxants are also used for pain management.[5] The use of these drugs needs professional support because they cause relevant adverse effects, such as tolerance and substance dependence.[5] Other undesirable effects can be listed: renal dysfunction, hypertension, bleeding, gastric ulcers, high blood glucose levels, and

immunosuppression.[6] In addition, according to the Center for Disease Control and Prevention of the United States, more than 70,000 individuals died from drug overdoses in 2017, of which 47,000 were due to the use of opioids.[7]

In general, the mechanisms of action of opioid drugs, including codeine, involve inhibition of adenylyl cyclase activity, leading to decreased cell permeability to the sodium ion. Additionally, K+ channels may open and voltage-gated Ca^{2+} channels may be inhibited. These events negatively affect the propagation of the nervous impulse.[8]

Codeine, usually in combination with acetaminophen, is the most commonly used opioid for the treatment of acute and moderate dental pain.[9] This information plays an important role in investigations into new therapeutic targets for pain treatment. Some plants and their metabolites are promising in the treatment of orofacial pain.[10] Perillyl alcohol (PA) is a monoterpene found in essential oils obtained from *Prunus cerasus*, *Lavandula augustifolia, Cymbopogon citratus, Zingiber officinale,* and *Apium graveolens.* Previous studies have reported PA has antitumor, anti-inflammatory, antioxidant, and antinociceptive activities.[11,12]

Our research group has found significant orofacial antinociceptive effect of PA in mice.[13] The mechanisms of action of PA are not yet elucidated, but they may involve opioid, vanilloid (TRPV1), and/or N-methyl-D-aspartate (NMDA) receptors.[13] This finding led us to hypothesize that PA associated with codeine might have a similar or better effect than does codeine alone, reducing the required dose of opioid analgesic. Thus, the aim of this study was to investigate the orofacial antinociceptive effects of PA associated with codeine in mice by investigating the pain pathways induced by formalin, capsaicin, and glutamate. Additionally, the mechanisms of action were evaluated by means of a molecular docking study.

Methodology

Animal study

This study adopted an experimental mouse model design, characterized as controlled, randomized, double-blind, and non-clinical investigation. The research project was previously approved by the Animal Research Ethics Committee of the Federal University of Paraíba (process n°. 1482130318). Adult male albino Swiss mice (*Mus musculus*) were used. They were subjected to a 12-hour light-dark cycle (light: 6:00 a.m. to 6:00 p.m.) and fed a balanced diet with water *ad libitum*. Animals were transferred to experimental room conditions 60 min before the start of the experiment, aiming to minimize behavioral changes associated with the new environment.

For sample size calculation, the expected effect size was obtained from a previous study.[13,14] Using a 5% significance level in a two-tailed test, Hedge's g of 2.99 (effect size), based on data from a previous study [(control, mean 84.4 s (SD ± 15.4), and PA, mean 45.3 s (SD ± 10.4)], a statistical power of 90%, and sample loss of 25%, the required sample size was five mice per group, totaling 75 animals.

Substances and experiments

The orofacial antinociceptive activity of S-(-)-perillyl alcohol was tested using 75 animals divided into three test groups. The experimental and control groups were formed considering different substances administered and doses used (Table 1). Three tests were performed to evaluate the orofacial antinociceptive activity of PA associated with codeine: a) formalin-induced nociception, b) capsaicin-induced nociception, and c) glutamate-induced nociception.

For all the test groups (formalin, capsaicin, and glutamate), the animals were pretreated with (S)-(-)-perillyl alcohol (Sigma-Aldrich®, St. Louis, USA) associated with codeine (Sigma-Aldrich®, St. Louis, USA), or vehicle [water and 0.2% Tween 80 (Vetec ®, Rio de Janeiro, Brazil)] by intraperitoneal injection for 60 min before administration of nociceptive substances. The doses used are described in Table 1.

Nociception was induced by 2% formalin (20 µL, ÊxodoCientífica®, São Paulo, Brazil), capsaicin (20 µL, 2.5 µg – Sigma-Aldrich®: St. Louis, USA), and glutamate (40 µL, 25 µM – Sigma-Aldrich®: St. Louis, USA) injected into the right upper lip of the mice (paranasal or vibrissa area) with a 27-gauge needle, according to a previously described protocol.[15] After the administration of nociceptive substances, the animals, individually, were immediately placed

Table 1. Substance names, doses used, and coding attributed to the experimental and control groups.

N	Substances	Dose	Coding
5	PA	75 mg/kg	PA75
5	PA + Codeine	75 mg/kg + 30 mg/kg	PA75/C30
5	PA + Codeine	37.5 mg/kg + 15 mg/kg	PA37.5/C15
5	Codeine	30 mg/kg	C30
5	Control	Saline + 0.2% Tween 80	Control

PA: Perillyl alcohol

in a mirrored box. Feeding interval analysis and spontaneous grooming behavior were considered. The orofacial nociception outcome was defined as the time (in seconds) during which the animal remained rubbing the orofacial region with its hind or front legs. A calibrated researcher used a stopwatch to check the grooming behavior intervals for each test individually.

Formalin-induced orofacial rubbing test

The formalin-induced orofacial rubbing test was performed as previously described.[16] Animals (n = 5) were pretreated with PA (75.0 mg/kg, IP), PA (75.0 mg/kg, IP) associated with codeine (30 mg/kg IP), PA (37.5 mg/kg, IP) associated with codeine (15 mg/kg IP), codeine (30.0 mg/kg, IP) or vehicle (saline + 0.2% Tween 80, IP) 30 min before the paranasal injection (20 µL) of 2.5% formalin., the animals were immediately placed individually in a transparent acrylic chamber for 30 min for observation of their behaviors. The formalin test consisted of a neurogenic phase (0–5 min) and an inflammatory phase (15–40 min).[17]

Capsaicin-induced orofacial rubbing test

This test involved the participation of TRPV1 receptor in orofacial nociception.[18] The mice (n = 5) were pretreated with PA (75.0 mg/kg, IP), PA (75.0 mg/kg, IP) associated with codeine (30 mg/kg IP), PA (37.5 mg/kg, IP) associated with codeine (15 mg/kg IP), codeine (30.0 mg/kg, IP), or vehicle (saline + 0.2% Tween 80, IP) 30 min before capsaicin injection (20 µL, 2.5 µg) into the paranasal region. The animals were immediately placed individually in a transparent acrylic chamber for 5 min for observation of their behaviors.[15]

Glutamate-induced orofacial rubbing Test

Prior to the evaluation of glutamate-induced nociception, the mice (n=5) were treated with PA (75.0 mg/kg, IP), PA (75.0 mg/kg, IP) associated with codeine (30 mg/kg IP), PA (37.5 mg/kg, IP) associated with codeine (15 mg/kg IP), codeine (30.0 mg/kg, IP), or vehicle (saline + 0.2% Tween 80, IP) 30 min before glutamate (40 µL, 25 µM) injection into the paranasal region. Subsequently, the animals were placed, one at a time, in a transparent acrylic chamber for 15 min for observation of their behaviors. This test was conducted, with slight modifications, according to a previous study.[19]

Data management and blinding procedure

To minimize bias associated with observation and data analysis, the samples were allocated randomly to study groups, and double-blinding of the examiners was applied. The examiner who performed the statistical analysis also did not know about the administered treatment.

Molecular analysis – *in silico* study

Perillyl alcohol structure was obtained from *PubChem* (https://pubchem.ncbi.nlm.nih.gov/compound/637566#section=Top), in *sdf* format. The receptors were obtained from the *Protein Data Bank – PDB* (http://www.rcsb.org/). In this study, receptors associated with nociception were selected.[20-22]

Initially, the receptors and their ligands were obtained: metabotropic glutamate receptor (PDB ID: 1S50, glutamic acid ligand), NMDA (PDB ID: 2A5S, glutamic acid ligand), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (PDB ID: 3DLN, glutamic acid ligand), mu-opioid receptor (PDB ID: 4DKL, morphine ligand), kappa opioid receptor (PDB

ID: 4DJH, JDTic ligand), delta-opioid receptor (PDB ID: 4EJ4, naltrindole ligand), and transient receptor potential vanilloid subtype 1 (PDB ID: 5IRZ, C24 H45 O13 P ligand).

PA was subjected to molecular anchorage using *Molegro Virtual Docker* v. 6.0.1 software.[23] The receptors were entered into the software and their ligands were excluded to permit binding to PA. PA was then entered into the software and the water molecules were deleted from the enzymatic structure. The binding between ligand and receptor was measured in terms of affinity energy value (Kcal/mol), and the more negative the value, the better the binding.

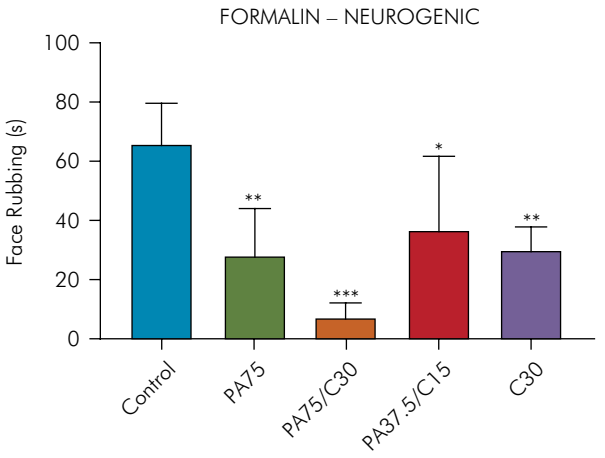
Statistical analysis

The statistical analysis was performed using equations available elsewhere.[14] A 5% level of significance was considered for the two-tailed test. Normality of data was confirmed by verifying that both skewness and kurtosis were within the acceptable range (-2 to 2). First, one-way ANOVA was used to test the difference among all five groups. Pairwise comparisons were then performed using a t-test for heterogeneous variances (after homogeneity of variances was excluded by identifying that the ratio between the highest and the lowest variances exceeded 3).

Results

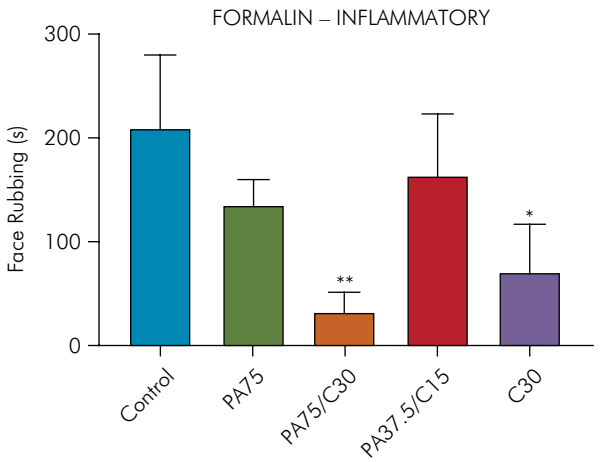
Formalin-induced nociception permits verifying the effects of antinociceptive substances in the neurogenic and anti-inflammatory phases. In the neurogenic phase, PA 75/C 30 ($p = 0.000007$) and PA 37.5/C 15 ($p = 0.04$) revealed a reduction in nociceptive behavior when compared with the control group. Besides, PA (75 mg/kg) associated with codeine (30 mg/kg) showed greater reduction in nociceptive behavior than that of the positive control, codeine ($p = 0.0005$). Another interesting finding was the similar effect of PA (75 mg/kg) and codeine (30 mg/kg) ($p = 0.8$). These findings are shown in Figure 1.

In the anti-inflammatory phase of the formalin test, PA 75/C 30 ($p = 0.002$) showed a promising effect. As observed in Figure 2, the association of PA with codeine showed a better orofacial antinociceptive effect than that of the positive control ($p = 0.012$).



Control (0.2% Tween 80); Perillyl alcohol (PA 75 mg/kg, IP); Perillyl alcohol associated with codeine (PA 75 mg/kg-C30 mg/kg); Perillyl alcohol associated with codeine (PA 37.5 mg/kg-C15 mg/kg IP); codeine (30 mg/kg, IP). Values expressed as means and standard deviation (n = 5, per group). *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$ versus control (One-way ANOVA and unpaired t-test of heterogeneous variances).

Figure 1. Effect of perillyl alcohol associated with codeine in the neurogenic phase of orofacial nociception induced by formalin.



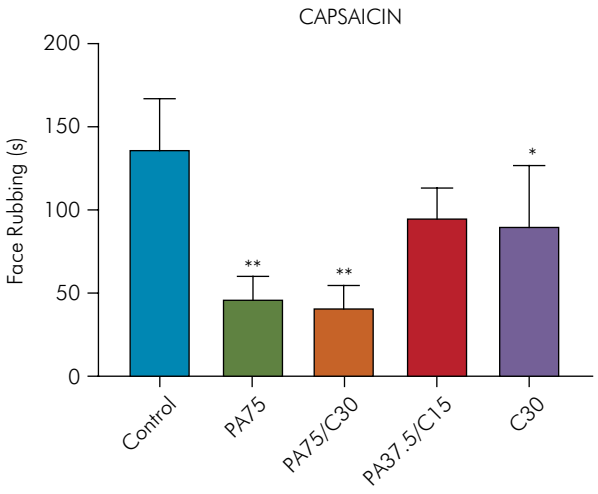
Control (0.2% Tween 80); Perillyl alcohol (PA 75 mg/kg, IP); Perillyl alcohol associated with codeine (PA 75 mg/kg-C30 mg/kg); Perillyl alcohol associated with codeine (PA 37.5 mg/kg-C15 mg/kg IP); codeine (30 mg/kg, IP). Values expressed as means and standard deviation (n = 5, per group). *$p < 0.0005$; **$p < 0.0001$ versus control (One-way ANOVA and unpaired t-test of heterogeneous variances).

Figure 2. Effect of perillyl alcohol associated with codeine in the inflammatory phase of orofacial nociception induced by formalin.

In the capsaicin test, as shown in Figure 3, PA 75/C 30 (p = 0.0005) and PA 75 (*P* = 0.04) presented a reduction in nociceptive behavior when compared with codeine (positive group). Furthermore, PA 37.5/C 15 demonstrated a similar orofacial antinociceptive effect to that of codeine (p = 0.78).

Glutamate-induced orofacial nociception was lower for PA 75/C 30 (p = 0.0005) and PA 37.5/C 15 (p = 0.001) when compared with the negative control. However, PA (75 mg/kg) alone showed orofacial antinociceptive activity similar to that of codeine (p = 0.46). These findings are described in Figure 4.

The possible interaction of PA with nociceptors was evaluated using the molecular anchorage method, and the binding affinity energies are shown in Table 2. High values of negative binding affinity energies were observed, especially in glutamate receptors (AMPA, MGLUR6, and NMDA). Predominance of steric effect and hydrogen bonds between PA and its receptors was observed (Figure 5).



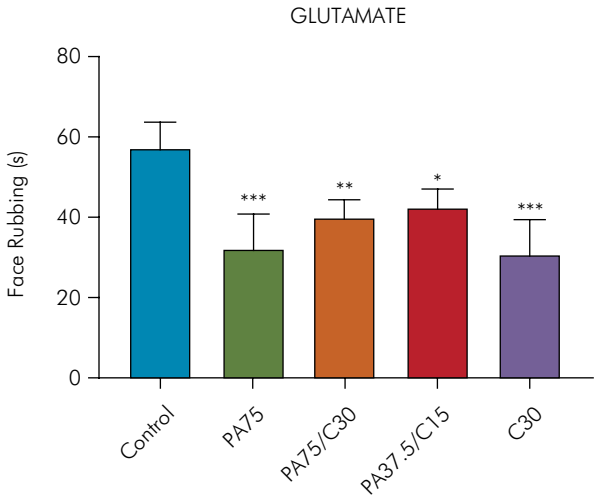
Control (0.2% Tween 80); Perillyl alcohol (PA 75 mg/kg, IP); Perillyl alcohol associated with codeine (PA 75 mg/kg-C30 mg/kg); Perillyl alcohol associated with codeine (PA 37.5 mg/kg-C15 mg/kg IP); codeine (30 mg/kg, IP). Values expressed as means and standard deviation (n = 5, per group). *p < 0.05; **p < 0.0001 versus control (One-way ANOVA and unpaired t-test of heterogeneous variances).

Figure 3. Effect of perillyl alcohol associated with codeine in orofacial nociception induced by capsaicin.

Discussion

The findings of this study indicate the administration of PA associated with codeine reduces the orofacial nociceptive response induced by formalin, capsaicin, and glutamate, which are recognized as relevant substances for the study of new antinociceptive drugs.[24]

Although some studies have shown antinociceptive effect of PA or essential oils with high concentrations



Control (0.2% Tween 80); Perillyl alcohol (PA 75 mg/kg, IP); Perillyl alcohol associated with codeine (PA 75 mg/kg-C30 mg/kg); Perillyl alcohol associated with codeine (PA 37.5 mg/kg-C15 mg/kg IP); codeine (30 mg/kg, IP). Values expressed as means and standard deviation (n = 5, per group). *p < 0.05; **p < 0.01; ***p < 0.0001 versus control (One-way ANOVA and unpaired t-test of heterogeneous variances).

Figure 4. Effect of perillyl alcohol associated with codeine in orofacial nociception induced by glutamate.

Table 2. Binding energies between perillyl alcohol and its receptors.

Receptors	Binding energy
AMPA	-79.57 Kcal/mol
Delta	-42.45 Kcal/mol
Kappa	-42.45 Kcal/mol
mGlur6	-71.25 Kcal/mol
Mu	-27.74 Kcal/mol
NMDA	-66.33 Kcal/mol
TRPV1	-53.34 Kcal/mol

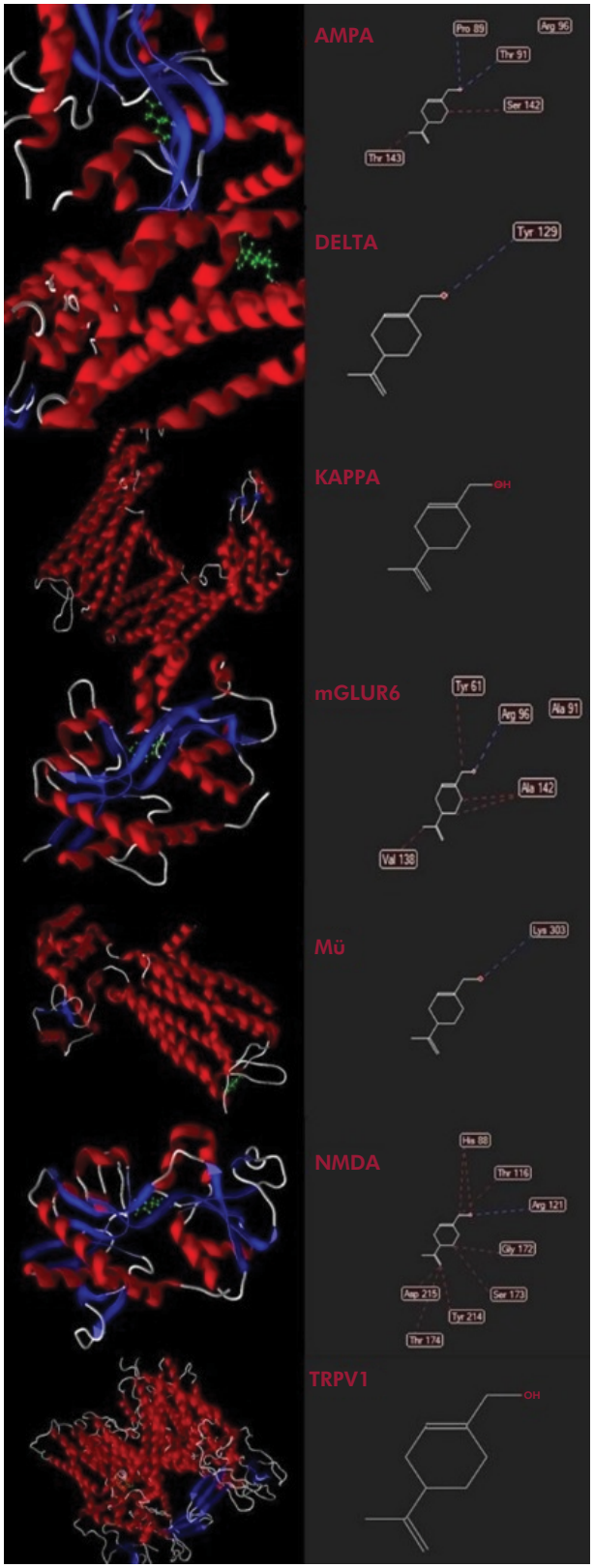


Figure 5. Interaction between perillyl alcohol (green) in the active site of receptors *in silico* and their interactions. Blue, red, and green are hydrogen, steric, and electrostatic binding, respectively.

of this molecule,[25,26] this is the first study to report orofacial antinociceptive activity for the association of PA with codeine, a drug used to treat orofacial pain, especially in acute situations. The proposed association of PA with codeine aims to make it possible to decrease the amount of opioid analgesic administered, minimizing possible undesirable effects.

In addition to antinociceptive activity, PA presents low toxicity and has been tested in humans in clinical trials (phases 1 and 2) aimed at evaluating its anticancer activity.[26-28]

Molecular anchorage studies show the possible molecular interactions between the ligand and its receptor, helping to understand its biokinetics and biomechanics and possible mechanism of action. It is a predictive model that assists in the evaluation of the pharmacological activity of a substance based on *in vivo* tests.[29,30]

The choice of models for the analysis of orofacial antinociceptive effect in this study considered neuroanatomical aspects of capture, path, and processing of information about the face region, more specifically about the trigeminal nerve. This model was used for behavior analysis in inflammatory conditions: changes in spontaneous grooming behavior.[31]

Formalin, when administered in the upper lip region of the animal, promotes a face rubbing behavior. This test consisted of two phases: the first or neurogenic phase (0–5 minutes) and the second or inflammatory phase (15–40 minutes). The biphasic component of this test indicates two different mechanisms. The first phase corresponds to the chemical stimulation of free nerve endings (C fibers), mediated by neuropeptides such as substance P. The second phase shows interactions between the central and peripheral nervous systems, with the release of excitatory amino acids, nitric oxide, and peptides.[15]

The administration of PA associated with codeine decreased orofacial nociceptive activity. This effect was observed in the two phases, indicating possible mechanisms of action involving nociceptive receptors and inflammatory mediators. The anti-inflammatory activity of PA can be associated with suppressed production of proinflammatory cytokines, such

as IL-1β, TNF-α, and IL-6. Moreover, this molecule down-regulates the expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (NOS2), and nuclear factor-kB.[12]

Although the *in silico* study has indicated lower values of negative energy between PA and opioid receptors when compared with glutamate receptors, PA allowed reversing the nociceptive effect exerted by formalin. Formalin is known for its nociceptive activity modulated by the participation of opioid receptors. Accordingly, further *in vivo* studies should be conducted to test the use of naloxone (an opioid receptor antagonist drug) to investigate one of the likely mechanisms of action.[32]

PA alone and associated with codeine reduced orofacial nociception induced by capsaicin, but the drug combination did not show synergistic activity, which is observed when the effect promoted by a drug combination is superior to the activity promoted by isolated substances.[24] Capsaicin-induced nociception involved the participation of TRPV1.[33] This information supports the finding obtained in the docking analysis, which showed affinity of PA for this receptor.

In the glutamate test, the association of PA with codeine (PA 75/C 30 and PA 37,5/C 15) did not reduce orofacial nociceptive activity. This effect can be explained by the drug combination, which can either increase or decrease the bioavailability of the substances.34 PA alone (75 mg/kg) promoted orofacial antinociceptive activity, as observed in a previous study.[13]

Glutamate is the amino acid more frequently found in the central nervous system and it is recognized as an important excitatory neurotransmitter involved in the nociceptive stimulus through activation of glutamate receptors.[35] Glutamate can bind to metabotropic or ionotropic receptors. When activated, metabotropic receptors (mGlur) promote the modulation of AMPc (second messenger) and the consequent opening of the sodium ion channel and propagation of nervous impulses.[36]

Ionotropic receptors, such as NMDA and AMPA, are responsible for fast synaptic transmission. When activated, they promote the influx of calcium (opening of Ca^{2+} ion channel) and consequent cell membrane depolarization. These receptors also release nitric oxide (NO).[36] The molecular anchorage study (molecular docking) provided evidence of the predilection of PA for glutamate receptors. High negative binding values mean more stable binding, especially to AMPA, mGlur6, and NMDA.

As expected, codeine reduced orofacial nociception in all tests performed. This substance is recognized for its effectiveness in acute pain relief, including dental pain, and its mechanism of action involves a pathway that is similar to that of other opioids.[9,37] Therefore, the orofacial antinociceptive effect promoted by PA associated with codeine may be mainly due to the action of these substances on opioid and glutamate receptors.

The findings of this study allow for the formulation of new hypotheses relative to a new association of PA and lower doses of opioid analgesics and for the proposition of pharmaceutical devices that could improve the bioavailability of drugs applied locally.

Conclusion

The association of PA with codeine showed orofacial antinociceptive activity induced by formalin, capsaicin, and glutamate nociception pathways, with theoretical evidence of greater chemical interaction with glutamate receptors.

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