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ATIVIDADE ANTINOCICEPTIVA OROFACIAL DO (*S*)-(-)-ÁLCOOL PERÍLICO EM CAMUNDONGOS: um estudo controlado, randomizado e triplo-cego

James Felipe Tomaz de Morais

2015

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Orientador: Prof. Dr. Ricardo Dias de Castro

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Banca Examinadora

Prof. Dr. Ricardo Dias de Castro

Titular 1 – Examinador Interno (Orientador)
Programa de Pós-graduação em Odontologia da Universidade Federal da Paraíba

---

Prof. Dr. Paulo Rogério Ferreti Bonan

Titular 2 – Examinador Interno
Programa de Pós-graduação em Odontologia da Universidade Federal da Paraíba

---

Profa. Dra. Edja Maria Melo de Brito Costa

Titular 3 – Examinador Externo
Programa de Pós-graduação em Odontologia da Universidade Estadual da Paraíba

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I don't believe you have to be better than everybody else. I believe you have to be better than you ever thought you could be.

Ken Venturi

RESUMO

Os monoterpenos são os principais componentes bioativos encontrados em óleos essenciais de plantas com uso medicinal para o tratamento de doenças. Dentre eles, o (*S*)-(-)-álcool perílico (AP) representa uma molécula promissora com atividades antitumoral, anti-inflamatória e antioxidantes. O presente estudo investigou os efeitos antinociceptivos do AP na nocicepção orofacial em camundongos suíços utilizando os testes de dor induzida por formalina, capsaicina e glutamato. Para cada teste, oito animais por grupo foram pré-tratados via intraperitoneal (i.p.) por um investigador cego com AP (50 e 57 mg/kg, i.p.), morfina (5 mg/kg, i.p.) ou veículo (salina + Tween 80 0.2%). O tratamento foi realizado trinta minutos antes da indução da nocicepção orofacial através da injeção de uma solução de formalina (20 µl, 2%), capsaicina (20 µl, 2.5µg) ou glutamato (40 µl, 25 mM) no lábio superior direito do animal utilizando uma agulha calibre 27G. O tempo de comportamento de nocicepção orofacial foi medido por um investigador cego para os grupos de tratamento, assim como a análise estatística. Os grupos foram comparados utilizando o teste de Mann-Whitney e a correlação entre as doses foi calculada pelo teste de correlação de Spearman, descrevendo para cada grupo os valores de mediana e distância interquartil. A magnitude da análise estatística foi verificada com os intervalos de confiança, magnitude do efeito e poder. Os resultados indicaram que o AP bloqueou o comportamento de nocicepção orofacial em todas as doses testadas ($p < ,05$) de modo similar à morfina ($p < ,05$) nos testes da formalina, capsaicina e glutamato. A magnitude do efeito foi alta para a fase 1 do teste da formalina nas doses de 50 e 75 mg/kg de AP (IC95%: 2,32/0,48; poder: 84% e IC95%: 2,76/0,82; poder: 96,2%, respectivamente), assim como na dose de 75 mg/kg na fase II do teste da formalina (IC95%: 2,26/0,44; poder: 82,3%) e no teste do glutamato (IC95=3,16/1,11; poder: 99,2%). Os testes com AP confirmam uma forte evidência de sua atividade antinociceptiva considerando os altos valores de poder principalmente nos modelos da formalina e glutamato, sendo assim uma substância potencial para o tratamento de condições clínicas envolvendo dor orofacial.

Palavras-chave: álcool perílico, monoterpenos, analgésicos, dor orofacial, experimentação animal, óleos essenciais, produtos naturais

ABSTRACT

Monoterpenes are the major bioactive compounds found in essential oils from medicinal plants used in the treatment of diseases. Among these, (*S*)-(-)-perillyl alcohol (PA) stands as a promising molecule with antitumor, anti-inflammatory and antioxidant properties. This study investigated the antinociceptive effects of PA on orofacial nociception in Swiss male mice using tests of formalin-, capsaicin-, and glutamate-induced pain. For each test, eight animals per group were pretreated intraperitoneally by a blinded investigator with PA (50 and 75 mg/kg, i.p.), morphine (5 mg/kg, i.p.) or vehicle (saline + 0.2% Tween 80). The treatment was performed thirty minutes before the induction of orofacial nociception by injecting formalin (20 µl, 2%), capsaicin (20 µl, 2.5 µg) or glutamate (40 µl, 25 mM) solution in the right area of the upper lip. The orofacial nociceptive behavior was timed in all tests by an investigator blinded to the treatments. Statistical analysis was performed by a blinded researcher. Groups were compared with Mann-Whitney's test and the correlation was calculated with Spearman's correlation test, describing its median and interquartile range. The magnitude of statistical analysis was also analyzed with confidence intervals, effect size and power. The results indicate that PA blocked the orofacial nociceptive behavior at all tested doses ($P < .05$) similarly to morphine ($P > .05$) in the formalin, capsaicin and glutamate tests. Effect size was high in phase I of formalin test for 50 mg/kg and 75 mg/kg of PA (CI95%: 2,32/0,48; power: 84% and CI95%: 2,76/0,82; power: 96.2%, respectively), 75 mg/kg of PA in phase II (CI95%: 2,26/0,44; power: 82.3%) and for 75 mg/kg of PA in glutamate test (CI95=3,16/1,11; power: 99.2%). These findings confirm a strong evidence of antinociceptive properties of PA in the orofacial region considering high values of power observed in formalin and glutamate models, suggesting it as a potential substance for the treatment of clinical conditions involving orofacial pain.

Keywords: perillyl alcohol, monoterpenes, analgesics, orofacial pain, animal experimentation, essential oils, natural products

LISTA DE ABREVIATURAS E SIGLAS

AINES – anti-inflamatórios não-esteroidais, NSAIDS – *nonsteroidal anti-inflammatory drugs* (Inglês)

AMPA - alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico

AP – (*S*)-(-)-álcool perílico (Português), PA – (*S*)-(-)-*perillyl alcohol* (Inglês)

DIQ – Distância inter-quartil (Português), IQR – *Interquartile range* (Inglês)

DTM – disfunção temporomandibular

ES – *Effect size* (Inglês)

IC – Intervalo de confiança (Português), CI – *Confidence interval* (Inglês)

NMDA – N-metil-D-aspartato

NO – óxido nítrico

PASW – *Predictive Analytics Software* ®

PGE_2 – prostaglandina E2

UFPB – Universidade Federal da Paraíba

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1. INTRODUÇÃO

Dor orofacial é o termo dado às condições dolorosas na região facial sentidas por estruturas inervadas pelos ramos do nervo trigêmeo (V par de nervos cranianos). Dentre as condições com dor orofacial estão a disfunção temporomandibular (DTM), neuralgia trigeminal e síndrome da ardência bucal (1-3). Na face, as fibras nervosas responsáveis pela condução dos estímulos dolorosos são do tipo Aδ e C, pertencentes ao N. trigêmeo através de seus três ramos: oftálmico, maxilar e mandibular. O corpo dos neurônios está localizado no gânglio trigeminal, o qual recebe impulsos provenientes dos axônios na periferia da face. No tronco encefálico, esses neurônios fazem sinapses em neurônios de 2ª ordem de modo especial no complexo nuclear trigeminal espinal (incluindo o núcleo sensitivo e o núcleo do trato espinal do N. trigêmeo, este último dividido nos subnúcleos oral, interpolar e caudal) (2, 4).

A prevalência deste tipo de dor na população em geral é alta, alguns estudos apontam que cerca de 20% da população possui algum tipo de dor orofacial (5, 6). Os medicamentos podem ser aliados favoráveis no tratamento da sintomatologia dolorosa nos quadros de dor na face (7). A terapia farmacológica atual em dor orofacial inclui agentes analgésicos, anti-inflamatórios não-esteroidais (AINES), anestésicos locais, corticoesteróides injetáveis ou orais, relaxantes musculares (7) e mais recentemente, a toxina botulínica (8). Estas drogas devem ser prescritas com cautela pelos clínicos, considerando que seu uso continuado pode desenvolver tolerância e dependência farmacológicas (9). Além disto, atenção deve ser dada para as contra-indicações ou variações dos efeitos dependendo da região onde a dor é referida (10). O uso prolongado de AINES não é indicado devido à alta incidência de exacerbação da hipertensão e efeitos adversos nos sistemas digestivo e urinário (11); anestésicos locais

possuem eficácia variável além da inconveniência da administração (9); e alguns relaxantes musculares também já foram relatados com efeitos adversos.

Considerando as vias de administração utilizadas para a maioria dos fármacos para o tratamento da dor orofacial (intra-articular, intramuscular, etc.) (9) o uso desses agentes na clínica torna o processo terapêutico pouco conveniente.

Na linha de experimentos em dor orofacial, a vertente com aplicação de produtos de origem natural para o tratamento desta condição tem expressiva produção nos últimos anos. As plantas são grandes reservatórios de substâncias antinociceptivas, encontradas em flores, folhas e frutos cuja constituição destacam-se os óleos essenciais(12). Estes óleos são misturas de natureza complexa de substâncias líquidas, voláteis e lipofílicas, em geral odoríferas. Sua constituição química pode ser de hidrocarbonetos terpênicos, terpenoides, álcoois terpênicos simples, aldeídos, cetonas, fenóis, ésteres, peróxidos, furanos, ácidos orgânicos e cumarinas(13). Dentre os terpenoides mais comuns em óleos essenciais estão os monoterpenos, os quais são responsáveis por cerca de 90% de sua composição(14). Os terpenos observados em óleos essenciais possuem moléculas simples, de baixo peso molecular, e geralmente possuem potencial analgésico e alta solubilidade, permitindo que atravessem a barreira hematoencefálica e que atue no sistema nervoso central(14).

A recente perspectiva de utilização de outros agentes químicos toma destaque, impulsionando a realização de investigações de produtos de origem natural de ação antinociceptiva para dor geral e orofacial (15-23). Das substâncias com origem natural já descritas com atividade antinociceptiva orofacial estão o óleos essenciais de *Cymbopogon winterianus*(24) e *Lippia grata*(25), extrato etanólico de *Syzygium cumini*(26), *Hyptis fruticosa*(27) e *Acmella oleracea*(28), extrato de *Hyptis pectinata*(29) e *atranorin*(30), incluindo os terpenos o citronelol (19), citronelal(15), carvacrol(17), p-cimene(21) e alpha,beta-aminina (31).

O (*S*)-(-)-álcool perílico (AP), um monoterpeno encontrado em óleos essenciais de *cerasus, lavandula, mentha, cymbopogon citratus, zingiber officinale* e sementes de *apium graveolens* (32). Esta substância é mais conhecida na literatura por sua atividade antitumoral, a qual já foi testada em mais de 30 estudos utilizando diferentes mecanismos, modelos animais e linhagens celulares(33). Além desta, o AP ainda possui atividades anti-inflamatória e antioxidante bem descritas (34, 35). Baseado nessas propriedades, o AP surge como molécula promissora no tratamento da dor, uma vez que estudo prévio indicou sua atividade antinociceptiva(36), embora não tenha sido investigada em modelo de abordagem orofacial.

Poucos estudos utilizando modelos animais de nocicepção orofacial são conduzidos afim de compreender as interações de fármacos de finalidade analgésica com o sistema trigeminal, apesar do alto interesse clínico nas condições de dor orofacial.

Os modelos animais atuais para o estudo da atividade farmacológica de agentes anti-inflamatórios e antinociceptivos na região orofacial foram desenvolvidos ou adaptados para esta função e incluem os métodos Complete Freund's Adjuvant(37), carragenina(38), formalina(39, 40), capsaicina(41), glutamato(15, 42) e o método de constrição do N. infraobrital para dor orofacial crônica(43). Dentre os mais utilizados, estão os de dor orofacial não-dental induzida por agentes nociceptivo em via subcutânea (formalina, capsaicina e glutamato).

De modo geral, os resultados nesses modelos envolvem a comparação dos efeitos produzidos pela substância-teste e a substância padrão-ouro e controles. Os animais são submetidos ao tratamento seguido da indução da nocicepção orofacial por substâncias percussoras dos processos celulares envolvidos na transmissão nociceptiva. Os efeitos são analisados através dos comportamentos

espontâneos de nocicepção na região orofacial de estímulos táteis. Em roedores, o comportamento espontâneo observado é o de fricção (coçar) a região perinasal com as patas dianteiras ou traseiras, comportamento mais conhecido pelo seu uso em inglês, *grooming behavior(44, 45)*.

Quando comparada à pesquisa pré-clínica para condições dolorosas em outras partes do corpo, a região orofacial é aquela com alto interesse clínico com menor número de experimentos utilizando modelos animais. A dor na musculatura mastigatória e pericraniana é o principal componente da disfunção temporomandibular e da cefaleia do tipo tensional, além de outras alterações como dor de origem dental ou neuropática. A prevalência de DTM é considerada, em média, entre 6% a 12% da população (5), enquanto a cefaleia tensional possui média que varia entre 30% e 78% da população em geral (46). Por serem condições de alta prevalência as limitações na eficácia, os efeitos adversos e a utilização de métodos invasivos dos fármacos disponíveis torna evidente a necessidade de ampliação dos conhecimentos nesta área.

A literatura indica o (*S*)-(-)-álcool perílico como um agente promissor para o tratamento da dor orofacial, dado que um estudo demonstrou sua atividade analgésica no sistema espinal (36). Porém, considerando as diferenças nos padrões aferentes do sistema nervoso, a atividade antinociceptiva do AP no sistema trigeminal necessita ser investigada.

2. CAPÍTULO I

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Authors' full names without degrees:

JF Tomaz-Morais[1,2], RM Braga[3], Sousa FB[1,2], DP Sousa[3], LCSLM Pordeus[4], RN Almeida[3], RD Castro[1]

Authors' institutional affiliations including city and country:

1 Post-graduate program in Dentistry, Universidade Federal da Paraíba, Brazil.

2 Department of Morphology, Federal University of Paraíba, Brazil.

3 Post-graduate program in Natural Products and Bioactive Synthetics, Universidade Federal da Paraíba, Brazil.

4 Post-graduate program in Cognitive Neuroscience and Behavior, Universidade Federal da Paraíba, Brazil.

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Abstract: Monoterpenes are the major bioactive compounds found in essential oils from medicinal plants used in the treatment of diseases. Among these, (*S*)-(-)-perillyl alcohol (PA) stands as a promising molecule with antitumor, anti-inflammatory and antioxidant properties. This study investigated the antinociceptive effects of PA on orofacial nociception in Swiss male mice using tests of formalin-, capsaicin-, and glutamate-induced pain. For each test, eight animals per group were pretreated intraperitoneally by a blinded investigator with

PA (50 and 75 mg/kg, i.p.), morphine (5 mg/kg, i.p.) or vehicle (saline + 0.2% Tween 80). The treatment was performed thirty minutes before the induction of orofacial nociception by injecting formalin (20 µl, 2%), capsaicin (20 µl, 2.5 µg) or glutamate (40 µl, 25 mM) solution in the right area of the upper lip. The orofacial nociceptive behavior was timed in all tests by an investigator blinded to the treatments. Statistical analysis was performed by a blinded researcher. Groups were compared with Mann-Whitney's test and the correlation was calculated with Spearman's correlation test, describing its median and interquartile range. The magnitude of statistical analysis was also analyzed with confidence intervals, effect size and power. The results indicate that PA blocked the orofacial nociceptive behavior at all tested doses ($P < .05$) similarly to morphine ($P > .05$) in the formalin, capsaicin and glutamate tests. Effect size was high in phase I of formalin test for 50 mg/kg and 75 mg/kg of PA (CI95%: 2,32/0,48; power: 84% and CI95%: 2,76/0,82; power: 96.2%, respectively), 75 mg/kg of PA in phase II (CI95%: 2,26/0,44; power: 82.3%) and for 75 mg/kg of PA in glutamate test (CI95=3,16/1,11; power: 99.2%). These findings confirm a strong evidence of antinociceptive properties of PA in the orofacial region considering high values of power observed in formalin and glutamate models, suggesting it as a potential substance for the treatment of clinical conditions involving orofacial pain.

Keywords: perillyl alcohol, monoterpenes, analgesics, orofacial pain, animal experimentation, essential oils, natural products

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Introduction

The current pharmacological therapy in orofacial pain includes the use of analgesics and corticosteroids for acute conditions, and non-steroidal anti-inflammatory drugs (NSAIDs), local anesthetics and muscle relaxants for acute and chronic conditions (Leeuw 2008). These drugs should be prescribed with caution by clinicians as their continued use can lead to drug tolerance and dependence (Liu and Steinkeler 2013). Attention should also be drawn to contraindications or variation of effects depending on the region where pain is referred (Nasri-Heir et al. 2013). The long-term use of NSAIDs is not indicated in view of the high incidence of exacerbation of hypertension and side effects in the digestive (gastric ulcers) and urinary (osmotic imbalance) systems (Cairns 2010); local anesthetics have variable efficacy as well as inconvenience of administration (injection) (Liu and Steinkeler 2013); and muscle relaxants have also been reported to have side effects.

In this background, the perspective of using other agents with analgesic activity in the orofacial region takes importance, especially the investigations with natural products. (*S*)-(-)-perillyl alcohol (PA) is a monoterpene found in essential oils of many plants, such as *menthas, cerasus, citrus* and *Cymbopogon citratus* (Crowell 1999). A great number of reports in the literature have described the antitumor activity of this monoterpene, including *in vitro* and *in vivo* data (i.e. Chen et al. 2014; da Fonseca et al. 2011), in addition to anti-inflammatory and antioxidant properties (Imamura et al. 2014; Tabassum et al. 2015). PA stands as a promising natural agent for the treatment of orofacial pain, given that a previous study demonstrated analgesic activity in the spinal system (Benedito 2009). Nevertheless, despite the differences in the afferent pattern of the nervous system, the antinociceptive activity of PA in the trigeminal system remains to be investigated.

Unlike the spinal system, orofacial nociception conducted by the trigeminal nerve comes from organs of special senses that are not found in other parts of the body. The processing of facial nociception occurs at the level of the brain stem in the spinal trigeminal nuclear complex, particularly in the subnucleus caudalis (Bereiter et al. 2000; Sessle 2005). It involves different properties from those

observed in the neurons of the dorsal horn of the spinal cord in relation to the other parts of the body (Bereiter et al. 2000).

The trigeminal and spinal afferent systems have particularities in the processing of pain upon many sorts of injury, making the trigeminal system unique with regard to features that contribute to the responses to injuries when compared to the spinal afferents (Hargreaves 2011). Among the animal models developed or adapted for the study of orofacial pain with inflammatory and nociceptive agents are: Complete Freund's Adjuvant (Sugiyo et al. 2005), carrageenan (Yeo et al. 2008), formalin (Clavelou et al. 1989; Luccarini et al. 2006), capsaicin (Pelissier et al. 2002) and glutamate (Beirith et al. 2002).

The aim of this study was to investigate the orofacial antinociceptive effect of PA in mice using formalin, capsaicin, and glutamate animal models, considering the null hypothesis that: PA has not orofacial antinociceptive effect in animal models (no peripheric, neither central activity) and that there is no correlation in ordinal scale between different concentrations of PA and control substances (vehicle and morphine).

Material and methods

Experimental animals

This was a controlled, randomized, triple-blind study carried out with healthy adult Swiss albino male mice (*Mus musculus*) from the Vivarium Prof. Thomas George of the Universidade Federal da Paraíba (João Pessoa, Brazil). All animals weighed between 25 g and 35 g and were maintained in light-dark cycle of 12 hours under controlled temperature, with food and water *ad libitum*.

All research procedures were conducted according to the Brazilian Guidelines for the Care and Use of Animals for Scientific and Teaching Purposes of the National Council for the Control of Animal Experimentation and on ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments). This study was approved by the Ethics Committee on Animal Use of the Universidade Federal da Paraíba under protocol no. 0509/2014. A reduced number of animals were considered for the experiments, and all measures aiming to control their suffering

or stress after experimentation were undertaken in accordance with the Ethical Guidelines for Animal Research (Zimmermann 1983).

Sample size was estimated using the data of a pilot study using 16 animals. The sample size calculation determined that 8 animals per group provides 98% of statistical power to detect diferences in orofacial nociceptive behavior in mice (considering one-sided type I error of 0,01, ES g = 2,43) (Cohen 1988).

Induced orofacial nociception tests.

A total of three tests were performed to evaluate the orofacial antinociceptive activity of PA in mice: (i) formalin-induced nociception, (ii) capsaicin-induced nociception, and (iii) glutamate-induced nociception. Each test was performed using four distinct groups of eight animals each (n=32). The animals were pretreated with morphine (5 mg/kg; i.p.), PA (50 and 75 mg/kg, i.p.) or vehicle (0.2% Tween 80) with subsequent induction of orofacial pain. The test doses of PA were defined based on its activity for general pain (Benedict 2009). All test substances were purchased from Sigma Aldrich, Missouri, USA.

Blinding. In order to prevent data collection and analysis biases, randomization and blinding procedures were used during the experiments. As the treatment groups had the same sample size, a block randomization protocol was carried out prior to experiments as suggested by Suresh (2011) (Available online at www.randomization.com, accessed on April 31, 2015). Blinding was performed by designating arbitrary codes using the software Biostat 2009® for each test variable: (i) nociception test; (ii) treatment group; (iii) treatment substance and dosage; (iv) follow-up time; and (v) sample size. The categorization and codification of the animals was conducted by a researcher (A) prior to experiments. Another researcher (B), blinded to the substances, proceeded with the administration of treatments. After induction with the algogenic substance by a researcher A, the evaluation of nociception was performed by a researcher C, who had been trained and was blinded to the treatment groups.

Formalin-, capsaicin- and glutamate-induced orofacial nociception tests. The formalin test was initially proposed by Clavelou et al. (1989) to evaluate general pain induced in the plantar region; later on, it was adapted to orofacial

pain by Luccarini et al. (2006). The formalin test in the orofacial region is considered to be the only animal model of persistent cutaneous nociception in the trigeminal region. It allows assessing the magnitude of nociception generated by a long-duration chemical stimulus, in contrast with the majority of tests that involve a brief stimulus (Raboisson and Dallel 2004). The orofacial nociception was induced with the administration of 20 µl of a 20% formalin solution (Êxodo Científica, São Paulo, Brazil) in the right upper lip of the mice (paranasal or vibrissa region) with a 27G needle (Luccarini et al. 2006) 0.5 h after treatment. After administration of formalin, the animals were immediately placed in a mirrored box and another researcher, who had been trained and was blinded to the treatment groups, used a chronometer (Casio Co., Ltd., Japan) to record the time spent by the animals rubbing the injected region with its fore or hind paws. The measures of nociception behavior in this test is based on two distinct phases of formalin-activity (Quintans-Júnior et al. 2010): phase 1 or neurogenic (0-5 min) and phase 2 or inflammatory (15-40 min).

Another test, described by Pelissier et al. (2002) and adapted by Quintans-Júnior et al. (2010) consisted in the administration of 20 µl of a 2.5 µg solution of capsaicin (Sigma Aldrich, Missouri, USA) in the right upper lip of the mice, 0.5 h after treatment. Subsequently, the nociceptive behavior was timed by a blinded researcher for 42 minutes after induction of nociception.

Firstly described by Beirith (2002), the glutamate-induced nociception was adapted to the orofacial region. This last test consisted in the subcutaneous administration of 40 µl of a 25 µM glutamate solution (Sigma Aldrich, Missouri, USA) in the right upper lip of the mice with a 27G needle. After administration of glutamate, the animals were placed in an observation box and monitored to their orofacial nociceptive behavior for 15 minutes (Quintans-Júnior et al. 2010) by a blinded researcher.

Statistical Analysis

At the end of the experiments, the researcher A inserted the data into the software Predictive Analysis SoftWare 18 (PASW® for Windows; IBM Corp., Chicago, USA) and another researcher (D) performed a blind statistical analysis. Subsequently, all data were re-coded by researcher A.

Descriptive and inferential statistical analyses were carried out on PASW. The treatment groups were compared using Mann-Whitney test, with a 95% confidence level (α=.5%). The percentage of inhibition was calculated in Microsoft Excel 2013 for Windows using the formula (A-B) / A x 100, where A is the median for the control group and B is the median for the treatment groups (Franzotti et al., 2000). Additionally, hypothesis of linear association between treatment substances was verified with Spearman's test (α=.5%). The ES was calculated for both tests (Mann-Whitney and Spearman tests) using Hedge's g formula, described with its confidence intervals and power (Cohen 1988).

Results

Formalin test

The administration of PA (50 and 75 mg/kg) led to a significant decrease in the orofacial nociceptive behavior (*P* <.05) when compared to the negative control in the formalin test in both neurogenic and inflammatory phases (Figures 1 and 2). The statistical differences for the neurogenic phase had a high power with 84,6% (IC95%= 2,31/0,48) for 50 mg/kg and 96,2% (IC95%=2,76/0,82) for 75 mg/kg. This sample size was not able to detect differences between both doses tested of PA (Mann-Whitney test, *P* = 0,49, power 24,2%, IC95%= 1,32/-0,33), despite of occurrence of effect size comparing with control group. For the inflammatory phase, the 50 mg/kg and 75 mg/kg had a moderate and high power, respectively (power 73,6%, IC95%= 2,08/0,30 and power 82,3%, IC95%= 2,26/0,44).

In the neurogenic phase of the formalin test, the 50 mg/kg dose of PA reduced the nociceptive behavior by 51.76% and the dose of 75 mg/kg caused a reduction of 60.00%. The percentage of reduction of nociception in this phase using morphine was found to be 52.65%. An even greater inhibition was caused by PA in the inflammatory phase, with 72.05% and 83.33% reduction for the doses of 50 mg/kg and 75 mg/kg, respectively. Morphine decreased by 69.11% the nociceptive behavior.

Capsaicin test

A significant reduction of the capsaicin-induced orofacial nociceptive behavior was observed for the two tested doses of PA (*P* <.01) as compared with

the negative control (Figure 3). However, low effect size was detected comparing 50 and 75 mg/kg doses of PA with negative control group (power 43,3%, IC95%=1,62/-0,07 and power 53,6%, IC95%=1,76/0,04), respectively). In this test, both doses of PA and morphine were included in the same homogeneous subset with no significant differences between their medians (Mann-Whitney test, *P* > .05) (Figure 3).

For the capsaicin test, the PA (50 and 75 mg/kg) inhibited the orofacial nociceptive behavior by 62.30% and 72.43%, respectively, while morphine caused an inhibition of 80.08%.

Glutamate test

When compared to the control group, PA (50 and 75 mg/kg) caused a significant reduction in the orofacial nociceptive behavior induced by glutamate (*P* <.05) (Figure 4). In this test only the dose of 75 mg/kg had a high power of difference (power 99,2%, IC95%= 3,16/1,11). Moreover, only in this test, a high power was detected for 75 mg/kg when comparing to 50 mg/kg group (Mann-Whitney test, *P* = .02, power 97,8%, IC95%= 2,91/0,94).

The percentage of inhibition was 48.57% at 50 mg/kg and 71.43% at 75 mg/kg of PA, while morphine caused a reduction of 65.57%. The Mann-Whitney test showed no significant differences between medians of the other experimental groups (*P* > .05) (Figure 4).

The occurrence of a linear antinociceptive effect comparing treatment groups was additionally calculated for each test using Spearman's correlation test. The sum of all treatment groups for each test (n=32) was able to provide 95% of statistical power in order to detect an ordinal association between groups (vehicle, 50 and 75 mg/kg of PA and morphine). This sample size was verified considering one-sided type I error of .01. The treatment groups in each test (Formalin, capsaicin and glutamate) showed association (*P* < .01) with an ordinal decrease of orofacial nociceptive behavior with high power analysis. An acceptable power of association was found for inflammatory phase of formalin, capsaicin and glutamate tests. (Appendix).

Discussion

The mechanisms of reception and processing orofacial nociceptive stimuli mediated by components of the trigeminal nerve are well established. In humans, orofacial pain can be related to tissue inflammation, acute pulpitis, mucositis or even chronic conditions involving changes of the temporomandibular joint or mastication muscles (Sessle 2011). A number of studies have reported on the pharmacological properties of monoterpenes including their antinociceptive and anti-inflammatory activity in the orofacial region (Guimaraes et al. 2013; Guimarães et al. 2012; Quintans-Júnior et al. 2010), in addition to antitumor and antioxidant properties (Chen et al. 2014; da Fonseca et al. 2011; Imamura et al. 2014; Tabassum et al. 2015). There is only one study investigating the antinociceptive activity of isolated PA in animal models of formalin-induced general nociception and hot plate test (Benedict 2009) at doses of 50, 75 and 100 mg/kg. The lowest doses of PA that showed antinociceptive activity were selected to be tested in our study.

In the formalin test, the immediate chemical activation of nerve endings, particularly type C fibers, mediates the release of substance P (Raboisson and Dallel 2004). In response to that, a number of inflammatory mediators are released resulting in swelling, hyperemia and increased local temperature (Sessle 2005). This process is called neurogenic phase as the condition is generated by the nerves. Later on, the second phase results from the processing of different inputs of nociceptive afferents due to release of excitatory amino acids, prostaglandin E2 (PGE2), nitric oxide (NO), tachykinins, kinins and other peptides (Capuano et al. 2009). Moreover, recent evidence has demonstrated the involvement of peripheral interactions of substance P and that NK1 receptors take part in the process of hyperalgesia to heat associated with inflammation and persistent pain induced by formalin in the orofacial region (Teodoro et al. 2013).

Hyperalgesia caused by formalin injection is mediated by the glutamatergic system, in particular through N-methyl-D-aspartate (NMDA) receptors (Raboisson and Dallel 2004). Centrally acting drugs act in both phases of the test, while peripherally acting drugs (e.g. NSAIDs, inhibit only the second phase). Thus, our findings suggest that PA acts in both levels with no significant differences when

compared to morphine. The inhibition of the nociceptive behavior in both phases of the formalin test by the monoterpene citronellol has suggested an association with the blocking of voltage-dependent sodium channels (Brito et al. 2013). Taking into account the current findings this is likely to be the mechanism of PA (a monoterpene).

Thermal and chemical stimuli in the area supplied by the trigeminal nerve through fibers C and Aδ can be also detected by vanilloid receptors (TRVP1). A substance present in red pepper, capsaicin, can activate these receptors and generate a sensation of heat in neurons resulting in pain (Pelissier et al. 2002). The activation of TRVP1 receptor by capsaicin occurs due to the influx of cations which produces depolarization of the cell membrane and subsequent excitation (Pelissier et al. 2002). The transmission of the impulse resulting from this activation is done by a number of mediators, including tachykinins, substance P, excitatory amino acids, NO and other pro-inflammatory mediators (Waning et al. 2007). PA reduced capsaicin-induced orofacial nociception in mice, suggesting that it acts in capsaicin-binding sites thus preventing the nociception flow or inhibiting the transduction signal upon activation of the receptor.

The antinociceptive activity of PA was also observed in the glutamate test. Glutamate is an excitatory amino acid of the nervous system found in type C fibers, which is released from sensitized afferents upon injury or in response to noxious stimuli being transmitted from peripheral, spinal and supraspinal levels (Beirith et al. 2002). This action occurs synergistically with release of proinflammatory mediators and cytokines for the excitation of nerve fibers (Bernardino et al. 2005). According to our findings for the glutamate test, PA inhibited orofacial nociception at both doses similarly to morphine, indicating that it acts against the transmission of nociceptive information in the glutamatergic system through NMDA, kainate and alpha-amino-3-hydroxymethyl-5-4-isoxazolepropionic acid (AMPA) receptors (Beirith et al. 2002). Furthermore, the blockage of glutamate reuptake has been suggested as a new therapy in the treatment of chronic inflammatory pain conditions due to the effects in the inhibition of pain mediated by glutamate in nerve endings (Yang et al. 2015).

Glutamate is able to excite nociceptive afferents that supply the musculoskeletal tissues of the face, thereby generating pain. Such effect can be blocked with peripheral injection of an agonist of NMDA receptors. As this effect is similar to that of capsaicin it has been suggested that the excitability of peripheral afferents for some noxious stimuli in the orofacial region is controlled, at least in part, by interactions between NMDA and TRVP1 receptors (Sessle 2005). Taking into account the effects of both nociceptive agents, our findings suggest that PA acts on the mechanism of inhibition of these receptors decreasing neuronal excitability.

De Souza et al. (2009) suggested that depression of the central nervous system and non-specific muscle relaxing effect can reduce the motor coordination and invalidate the test results. Nevertheless, the doses of 50, 75 and 100 mg/kg of PA did not induce changes in motor coordination secondary to neurotoxic effects in mice in the Rota-rod test (Benedict 2009). Furthermore, the lethal dose D_{50} of PA was determined to be 281.3 mg/kg intraperitoneally (Benedict 2009) and its safety is confirmed with phase I clinical trials in humans investigating its antitumor activity (Morgan-Meadows et al. 2003; Stratton et al. 2008).

In this study, the investigators involved in the experimental testing and statistical analysis were blinded to the treatment groups, in order to prevent observer bias and ensure reliable evidence for future clinical trials in humans. Recent report (Hirst et al. 2014) have described the need for randomization and blinding as a way to reduce the risk of bias in animal research. This risk is the key point to consider blinding in trials as it can interfere considerably in the study results (Bello et al. 2014). In addition, two other points are critical in the use of these methods in animal research, as follows: to reduce the number of animals in research, and to avoid biased conclusions being taken as hypothesis in human research.

Thus, the results of the present study suggest a strong evidence for (*S*)-(-)-perillyl alcohol to have antinociceptive activity, considering the great values of power found in animal models for formalin and glutamate. PA appears as a potential substance to the development of formulations for the treatment of

conditions with orofacial pain. Further studies should determine the application profile of this agent, and its efficacy must be accessed with clinical trials.

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Figures, legends and tables

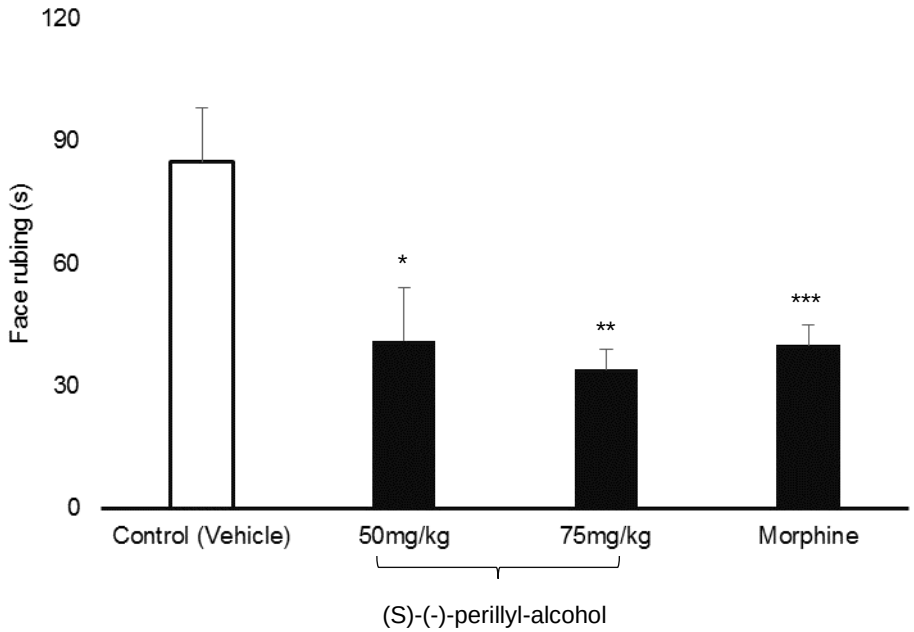

Fig. 1: Effects of PA on formalin-induced orofacial nociception (Phase 1). Vehicle, (S)-(-)-perillyl-alcohol (50mg/kg and 75mg/kg; i.p.) and Morphine (5 mg/kg; i.p.). Values expressed as median and IQD (n=8 per group).
*p=.019, **p=.001 e ***p=.007 *versus* negative control (Mann-Whitney test).

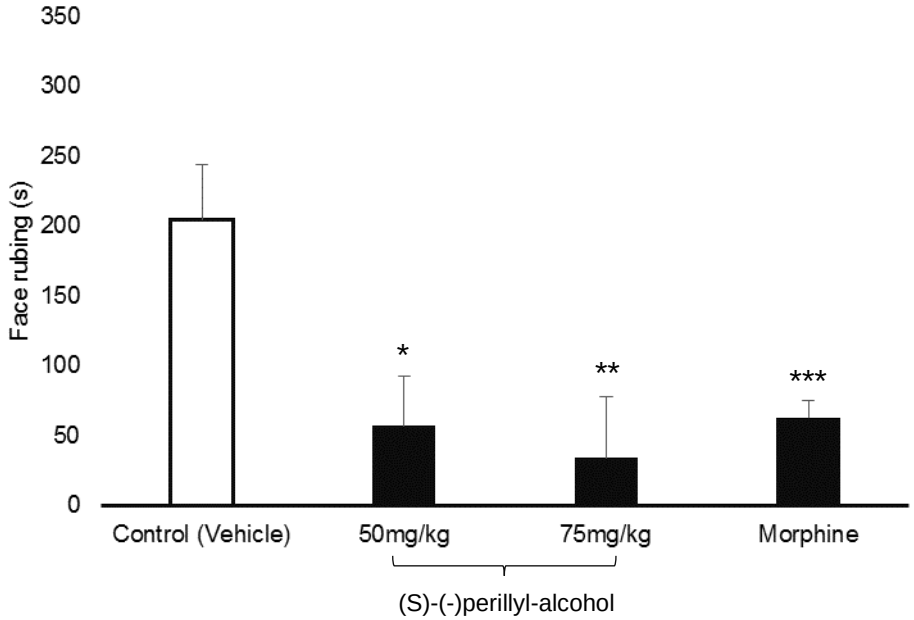

Fig. 2: Effects of PA on formalin-induced orofacial nociception (Phase 2). Vehicle, (S)-(-)-perillyl-alcohol (50mg/kg and 75mg/kg; i.p.) and Morphine (5 mg/kg; i.p.). Values expressed as median and IQD (n=8 per group).
*p=.013, **p=.007 e ***p=.005 versus negative control (Mann-Whitney test).

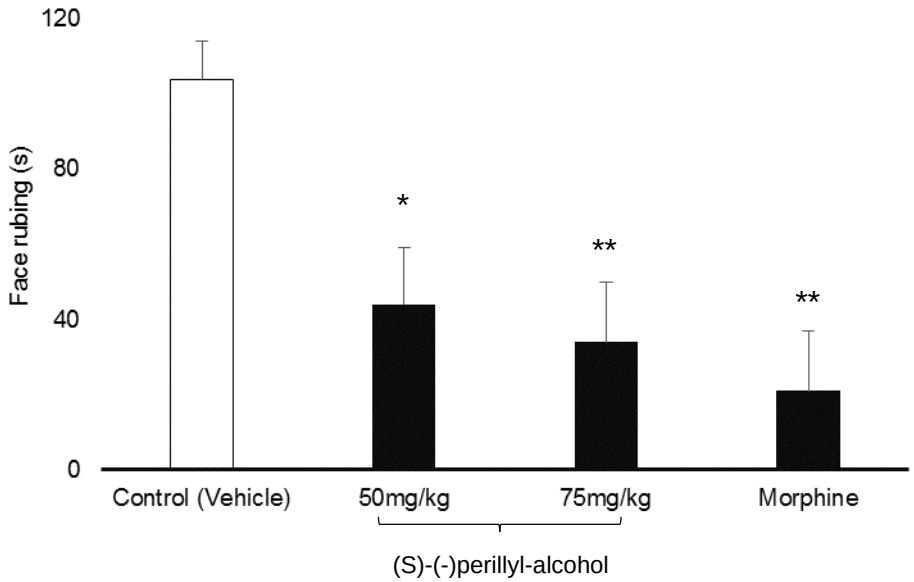

Fig. 3: Effects of PA on capsaicin-induced orofacial nociception. Vehicle, (S)-(-)-perillyl-alcohol (50mg/kg and 75mg/kg; i.p.) and Morphine (5 mg/kg; i.p.). Values expressed as median and IQD (n=8 per group).
*p=.016 e **p<.005 versus negative control (Mann-Whitney test).

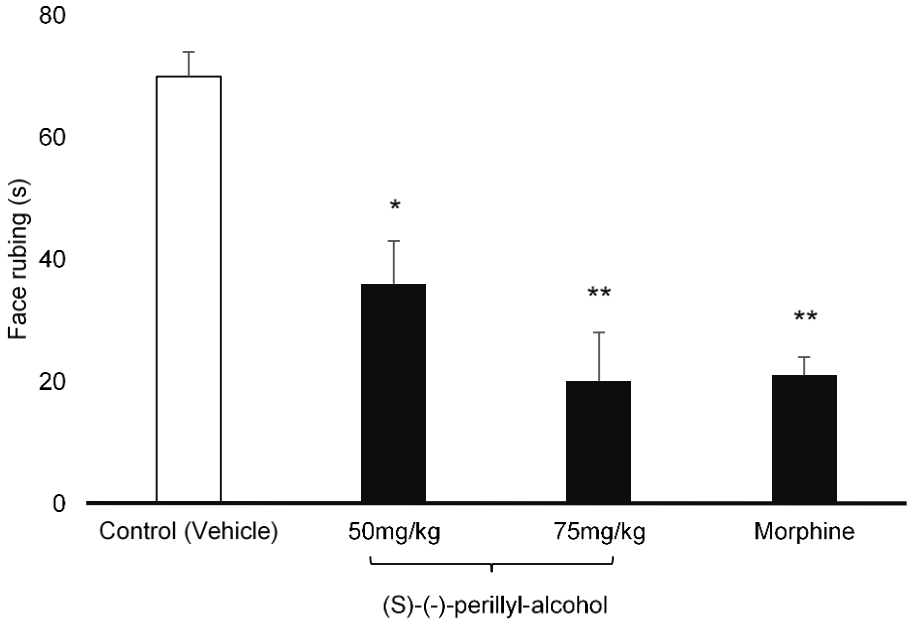

Fig. 4. Effects of PA on glutamate-induced orofacial nociception. Vehicle, (S)-(-)-perillyl-alcohol (50mg/kg and 75mg/kg; i.p.) and Morphine (5 mg/kg; i.p.). Values expressed as median and IQD (n=8 per group).
*p=.02 e **p=.001 versus negative control (Mann-Whitney test).

Appendix

Association hyphothesis tests of antinociceptive orofacial effects (n=32).

Induced orofacial nociception tests	**Effect size "r" (ES r)**	**CI 95% (superior/ inferior) of ES r ***	**Power ***	***P* value †**
Formalin (Phase 1)	0,415	0.63/0.14	77,3%	0,011
Formalin (Phase 2)	0,477	0.83/0.21	87,9%	0,0034
Capsaicin	0,54	0.73/0.27	93,4%	0,0018
Glutamate	0,497	0.70/0.21	88,2%	0,004

*Calculated with one-sided type I error of 5%; Statistical power: 95%.

† Spearman's correlation test.

3. CONSIDERAÇÕES GERAIS

Os mecanismos para captação e processamento dos estímulos nociceptivo orofaciais mediados pelos componentes do sistema trigeminal são bem estabelecidos. Em humanos, a dor orofacial pode estar relacionada a inflamação tecidual, pulpite aguda, mucosite e outras condições crônicas envolvendo alterações na articulação temporomandibular, músculos da mastigação ou o próprio nervo trigêmeo(47). Alguns estudos relataram as propriedades farmacológicas de uma série de monoterpenos incluindo sua atividade antinociceptiva e anti-inflamatória na região orofacial (15, 17, 48). Estudos em animais e em seres humanos, descrevem as propriedades antitumoral e antioxidante do (*S*)-(-)-álcool perílico (AP) (34, 35, 49-55), porém apenas um estudo investigou a atividade antinociceptiva deste monoterpeno isolado(36). A investigação desta propriedade utilizou modelos animais de dor geral induzida por formalina e o teste da placa quente com as doses de 50, 75 e 100 mg/kg de PA(36), sendo as menores doses com atividade antinociceptiva selecionadas para a realização do presente experimento

Modelos animais de experimentação possuem grande aplicabilidade à terapêutica da dor, em especial, por analisar as interações sistêmicas dos fármacos em teste e indicar a sua eficiência *in vivo.* Na região orofacial, os testes adquirem particularidades inerentes ao local e que não possuem similaridades em nenhum outro modelo, como por exemplo, os movimentos mandibulares envolvidos no ato de roer e mastigar. Nesta região, o comportamento espontâneo do roedor reproduz aspectos do ser humano nas condições de estimulação nociceptiva nas terminações nervosas trigeminais.

Os estudos laboratoriais e ensaios clínicos são fases distintas da experimentação de técnicas para o tratamento de doenças. Como o investigador é agente ativo no processo de pesquisa, é imprescindível que medidas de controle sejam tomadas para garantir o máximo de imparcialidade. A necessidade de planejamento de estudos laboratoriais com animais espelhados nos ensaios clínicos com humanos tem sido sugerida nos últimos anos(56, 57). Relatos recentes(56) descrevem a necessidade de

randomização e cegamento como uma estratégia para redução do risco de vieses na pesquisa animal, até então negligenciados, afim de evitar a super ou subestimação dos parâmetros observados incluindo a alocação aleatória dos animais. Este risco é o ponto chave para considerar o cegamento em ensaios pré-clínicos, já que o conhecimento da alocação pode interferir consideravelmente nos resultados do estudo(57). Desta forma, estas medidas foram consideradas incluindo a aleatoriedade, o tratamento e a avaliação de forma cega pelos pesquisadores. Dois aspectos fundamentais do controle metodológico foram considerados nesta pesquisa: o uso racional e reduzido de animais e a prevenção de que conclusões enviesadas sejam tomadas como hipóteses em ensaios clínicos com seres humanos.

Os monoterpenos tem sido considerados candidatos potenciais para o desenvolvimento de novas drogas com aplicações às condições clínicas com dor(48). Considerando o número de experimentos realizados com o (*S*)-(-)-álcool perílico (AP) para atividades antitumoral, anti-inflamatória e antioxidante (34, 35, 49-55), esta substância sustenta a sua potencialidade para o tratamento de doenças. Nesta pesquisa, foi possível identificar a capacidade do AP para bloquear a nocicepção na região orofacial em camundongos através dos modelos animais da formalina, capsaicina e glutamato. Desta forma, além das propriedades bem difundidas descritas anteriormente, confirmamos a propriedade antinociceptiva para o sistema trigeminal além do sistema espinal (36).

Considerando os valores de poder observados tanto nos testes da formalina e glutamato, quando o poder da correlação dos tratamentos em todos os testes, sugerem forte evidência da atividade antinociceptiva do AP. Os resultados atuais indicam a possibilidade de desenvolvimento de formulação com AP para o tratamento de condições com dor orofacial. Experimentos que esclareçam os mecanismos de ação e a farmacocinética do AP deverão ser realizados afim de potencializar o uso desta substância voltada a analgesia. Além disto, no futuro, estudos clínicos deverão determinar

o perfil de aplicação deste agente e seus usos na terapêutica através de ensaios clínicos.

4. CONCLUSÃO

Os resultados do presente estudo sugerem uma forte evidência da atividade antinociceptiva do (*S*)-(-)-álcool perílico, considerando os bons valores de poder encontrados nos modelos animais da formalina e glutamato. O AP aparece como substância potencial para o desenvolvimento de formulações no tratamento de condições com dor orofacial. Estudos futuros devem determinar o perfil de aplicação e administração deste agente, e sua eficácia deverá ser acessada com ensaios clínicos.

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APÊNDICES

Figura 1. Testes de nocicepção orofacial induzida

Teste	n	Substância de tratamento
	8	Controle (Veículo)
Formalina n=32	8	AP Dose-teste 1 (50 mg/kg)
	8	AP Dose-teste 2 (75 mg/kg)
	8	Morfina (5 mg/kg)
	8	Controle (Veículo)
Capsaicina n=32	8	AP Dose-teste 1 (50 mg/kg)
	8	AP Dose-teste 2 (75 mg/kg)
	8	Morfina (5 mg/kg)
	8	Controle (Veículo)
Glutamato n=32	8	AP Dose-teste 1 (50 mg/kg)
	8	AP Dose-teste 2 (75 mg/kg)
	8	Morfina (5 mg/kg)

Figura 2. Método de cegamento por codificação aleatória

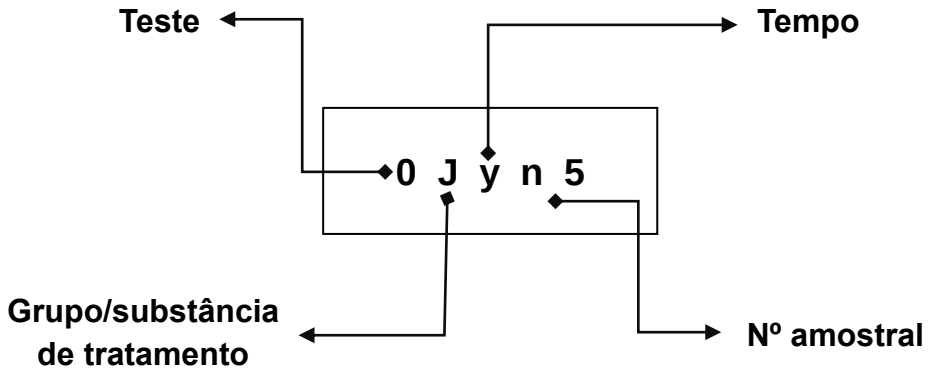

Figura 3. Plano de controle de vieses (cegamento)