

***In vivo* methods for the evaluation of anti-inflammatory and antinociceptive potential**

Métodos in vivo para avaliação do potencial anti-inflamatório e antinociceptivo

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ABSTRACT

BACKGROUND AND OBJECTIVES: The constant search for bioactive compounds with anti-inflammatory and antinociceptive activities are of interest to research centers. For the characterization of these activities, trials on guinea pigs are necessary. Therefore, the purpose of this study was to demonstrate some methods to evaluate the anti-inflammatory and antinociceptive potential of natural products.

CONTENTS: A stimulus is required to evaluate these activities, and the induction of inflammatory or nociceptive process can be by chemical inducers like formaldehyde, carrageenan, among others, or electronic equipment such as the hot plate. For all assays, the baseline and post-dose measurement of the studied compound is always compared with a control group. The planning of the experiment, as well as its conduct in accordance with well-established protocols, are important tools in the success of the work. The tests presented evaluated the antinociceptive and anti-inflammatory activity as well as the mechanisms involved.

CONCLUSION: It was possible to evaluate that the tests present in the literature today meet the researcher's need for the elucidation of the anti-inflammatory and antinociceptive activity of new compounds.

Keywords: Abdominal contortion, Formalin, Hot plate, Paw edema.

RESUMO

JUSTIFICATIVA E OBJETIVOS: A busca constante por compostos bioativos com atividade anti-inflamatória e antinociceptiva são de interesse dos centros de pesquisas. Para a caracterização dessas atividades são necessários ensaios em cobaias. Frente a isso, o objetivo deste estudo foi demonstrar alguns métodos para a avaliação do potencial anti-inflamatório e antinociceptivo de produtos naturais.

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CONTEÚDO: Para a avaliação dessas atividades é necessário um estímulo, sendo que a indução de processo inflamatório ou nociceptivo pode ser por indutores químicos como formol, carragenina, entre outros, ou ainda, equipamentos eletrônicos como placa quente. Para todos os ensaios, sempre é realizada a mensuração basal e posterior à administração do composto que está sendo estudado em comparação com um grupo controle. O planejamento do experimento, assim como toda a condução conforme protocolos já bem ilustrados, são ferramentas importantes no êxito do trabalho. Os testes apresentados avaliaram atividade antinociceptiva e anti-inflamatória assim como mecanismos envolvidos.

CONCLUSÃO: Foi possível avaliar que os testes presentes na literatura hoje, atendem a necessidade do pesquisador na elucidação da atividade anti-inflamatória e atividade antinociceptiva de novos compostos.

Descritores: Contorção abdominal, Edema de pata, Formalina, Placa quente.

INTRODUCTION

Natural products that carry biological activities are consumed daily around the world to help maintain human health, an ancient tradition that has been inherited for millennia. From popular knowledge, scientific investigation of the efficacy of these products is necessary¹. Among the diversity of natural products found, only a small portion have their phytochemical characterizations and biological potential investigated. The steps for clarification, such as isolation, identification, and obtaining structural analogues of the active metabolite, associated with knowledge of the pharmacological, toxicological potential and mechanism of action of these substances are fundamental to obtain and develop new therapeutic agents^{2,3}. The selection of active compounds present in natural products is a significant challenge faced by researchers, as is the elucidation of the mechanisms of action of these compounds. Discoveries require biological assays, which should be chosen with caution, since they need to be accurate in detecting the specific effect, have sensitivity and reproducibility⁴.

The amount of research carried out in search of compounds that are effective for application as an anti-inflammatory agent or with antinociceptive activity and which bring benefits with the fewest possible adverse effects caused by their use is well known. For the good conduct of the experiments, it is necessary the previous study of the methodologies that will be applied, as well as the tested doses and number of animals. One of the most commonly used tests for antinociceptive

evaluation is the formalin test. There were 395 articles published, and the anti-inflammatory activity is paw edema, with 244 articles, as evidenced by consulting the Pubmed database, in 2018 (until October).

Therefore, this study aimed to address the topics related to the main *in vivo* methods for the evaluation of new compounds with anti-inflammatory and antinociceptive potential, as well as the mechanisms involved.

CONTENTS

Due to the rejection rate of drugs available on the market due to the adverse effects, it is necessary to study new compounds with effective activities. Within ethnobotany, it is necessary to evaluate these activities, since the number of publications is increasing within this area. The selection of promising compounds within anti-inflammatory and analgesic activities begins with the culture of folk medicine followed by the chemical evaluation of these natural products. The literature mentions compounds such as alkaloids, essential oils, flavonoids, tannins, saponins, and phenolic compounds as responsible for the expected anti-inflammatory and analgesic activity, with greater effect than the drugs found in the market^{5,6}. To prove these activities, it is necessary to use animal models according to protocols, always respecting the ethical principles. All procedures must go through their own committee, responsible for the evaluation before the beginning of the experiments. Another important observation is the use of the smallest number of animals possible, correct application of the techniques, and the minimum suffering caused to the animal^{7,8}.

Animal selection

Animals such as *Mus musculus* mice, weighing on average 30±5g, and *Rattus norvegicus* rats weighing 150±20g on average are used as a model for *in vivo* testing. The choice of an animal model is based on the test performed and the expected result. It is advised to acclimate the animal for seven days, under controlled temperature conditions (25±3° C) and light/dark (12h) cycle with plenty food and water⁹.

Acetic acid-induced writhing tests¹⁰

The abdominal writhing test in mice is a widely used method to evaluate the analgesic activity of substances against pain of inflammatory origin, where the acetic acid (AA) in the concentration of 0.6% (0.1mL/10g of the animal) induces lesions on the abdomen of the mice, which is enough to cause the spasms translated as writhing. The compound being tested must be applied at the pre-defined concentrations. After 60 minutes, apply the peritoneal injection of AA and place the animal in an acrylic box so that it is possible to observe the number of contractions performed for 20 minutes¹¹.

Evaluation of analgesic and/or anti-inflammatory activity by formalin test¹²

The formalin test is a test performed to evaluate analgesia, which consists of the evaluation of the inflammatory process

at two moments: the so-called neurogenic phase and the inflammatory phase. The test is performed with mice receiving treatment 1h before the beginning of the test, along with negative control (vehicle), and positive control intraperitoneally (usually morphine, 2.5mg/kg b.w., i.p. administered 40 minutes before the analysis). The test begins with the intraplantar injection of 20µL of formalin at 2.5% in the right posterior pelvic limb and measuring the time the animal licks, shakes or bites the formalin-injected paw. From zero to five minutes are necessary to evaluate the painful sensitivity in the so-called neurogenic phase, in which the direct activation of nociceptors by the chemical agent occurs. Then, 15 to 30 minutes to determine pain sensitivity in the phase called inflammatory pain, which involves the spinal cord-reinforced synaptic transmission, as well as the release of local mediators such as prostaglandins and histamines¹³.

Formalin-induced orofacial pain. Adapted¹⁴

The formalin-induced orofacial pain test is performed to evaluate analgesia in the trigeminal nerve region of action where the literature describes several related diseases, leading to a mild to severe chronic pain. The test can be performed on rats or mice and consists of applying 50µL of the irritant substance, in this case, formalin at 2%, in the right cushion region of the animal's vibrissae. The application is subcutaneous, and the grooming process is evaluated by scratching the area where the formalin was applied to the front limbs, evaluating how often the animal performed the self-cleaning act, compared to controls^{15,16}.

Hot plate test¹⁷

The assay consists of exposing the animal to a hot surface for thermal stimulation to evaluate central mechanism-mediated analgesic activity. It is a model that assesses the antinociceptive activity of opioid drugs, but other centrally active drugs, such as sedatives and hypnotics, show activity in this experimental model¹⁸. The motor performance evaluation aims to detect the occurrence of motor incoordination, allowing a more accurate interpretation of the test results to determine the antinociceptive activity. Therefore, drugs that promote relaxation or sedation alter the motor performance and may interfere with the response, without necessarily being antinociceptive¹⁹. For this, a hot plate kept at 55°C should be used, where the animal will be placed during the time limit of 20s or until it flicks its paw to perform the act of licking (latency time). Measurements should be performed at zero, 30, 60, and 90 minutes after treatment. Along with the treated groups, one group with morphine (4.0mg/kg b.w.) should be included as the reference compound¹⁷.

Randall-Selitto test²⁰

It is used for nociceptive evaluation that tests the gastrocnemius muscle pressure. Pain measurement requires the use of an analgesiometer (Ugo-Basile, Stoelting, Chicago, IL). To perform the test, animals, usually rats, are allowed to rest in a dimly lit room with controlled temperature to reduce the

stress level. After 30 minutes, the animal has the lower pelvic limb placed on the equipment. After placing the animal, the equipment begins to apply pressure in grams, gradually, until the animal feels discomfort, flicking the limb or vocalizing. The equipment itself records the pressure supported in grams. The test should be performed before the administration of the drug tested and within 30 min, 1, 2, 3, and 4 hours, and compared with known analgesic drugs^{21,22}.

Von Frey test²³

The von Frey test, or rat paw gradually increase pressure test, consists of applying pressure in grams to the rat's hind limbs through electronic equipment. Initially, the described test used manual forms and has been changed to electronic forms for better and more accurate results; however, it is still possible to find work performed with manual equipment. The animal is positioned on the equipment, and through von Frey's rigid-tip monofilaments, the mechanical pain thresholds are applied and measured in grams. This test is used to evaluate the antinociceptive activity. The test is performed up to six times so that it is possible to obtain a measurement of three close paw flick values after applying a linear pressure. The result is quantified as the change in pressure (D reaction in grams) obtained by subtracting the average of three values expressed in grams (strength) observed before the experimental procedure (zero hour) from the average of three values in grams (strength) after the administration of the stimuli that vary according to the experiment²⁴.

Tail flick test²⁰

It is used to evaluate the antinociceptive activity promoted by the central nervous system and is simple to perform. The animal (rat) is placed on the equipment, and its tail rested on a spot where a beam of light will be focused that will heat the animal's tail. A baseline test should be performed, and animals with tail flick time longer than 7.9s should be excluded. After the administration of the evaluated natural product, perform the measurement within 2 hours (30, 60, 90, 120 minutes)^{19,25}.

Carrageenan-induced intraplantar edema²⁶

Intraplantar injection of carrageenan induces an acute and progressive increase in the volume of the injected paw. This edema, which is proportional to the intensity of the inflammatory response, is a useful parameter in the evaluation of the anti-inflammatory activity. Carrageenan triggers the inflammatory process mediated by prostaglandins, reaching peak levels between 2 and 3 hours after application^{27,28}. The inhibition of the edema caused by carrageenan involves the mechanism related to the prostaglandin synthesis, especially PGE₂ and PGF₂, and its activity is compared to non-steroidal anti-inflammatory drugs (NSAIDs)²⁹. Before testing, the volume of the animal's lower pelvic limb (hind paw) should be evaluated by plethysmometry. The administration should be in a vehicle control group to assess the solvent used for solubilization of the tested compound, a control group with an NSAID, and the test groups with well-defined doses. After 1h of the application, the volume should be measured at 30, 60, 120, and 180 minutes by the paw injection in the plethysmometer^{9,30}.

In addition to carrageenan³¹, which is the most used proinflammatory mediator, histamine³², dextran³³, xylene, and serotonin³⁴, bradykinin and prostaglandin can be used³⁵.

Croton oil-induced ear edema in mice³⁶

The importance of the test is to evaluate the ability to inhibit edema formation in the ear of the animals tested after the topical application of croton oil. To perform this test, the compound of interest should be administered one hour before on the surface of the inner ear. It should be decided which ear will receive the induction of the inflammatory process with croton oil, and which will receive acetone in the same quantity as the oil for the negative control. Measure the edema formation with the application of the compound, and in acetone. The test can also be used by adding a group of animals tested with drugs already known in the market, such as indomethacin and dexamethasone³⁷.

Carrageenan-induced peritonitis³⁸

It is possible to produce an inflammatory response, using carrageenan, in the peritoneal cavity with predominance of many polymorphonuclear cells present in the exudate. The treatment of the animals should be performed with the group of the vehicle used, a group with NSAID drug and test groups with well-defined concentrations. Apply carrageenan by intraperitoneal injection and wait for 4 hours for the animal to have an anti-inflammatory action and exudate formation. After the expected period, euthanize the animal and wash the peritoneum with heparinized PSB solution for polymorphonuclear cell counting. The number of leukocytes should be compared to the test group for analysis³⁹.

Pleurisy⁴⁰

In the pleurisy test, the systemic anti-inflammatory effect is evaluated through the exudate volume, that is, in an inflammatory process in the pleural region, the number of existing proteins tends to increase, and the number of defense cells (leukocytes) increases significantly⁴¹. In order to perform this study, a gavage treatment must be performed in the animals of the control group, with vehicle solution, a group with a known drug, and test groups with well-defined concentration, and after 60 minutes, induce the inflammatory process with carrageenan injection into the pleural region. Six hours after the induction of the inflammatory process, the animals should be euthanized, and the pleural cavity opened. Rinse with physiological solution and EDTA (Ethylenediaminetetraacetic acid) and perform cell count in a Neubauer chamber, always comparing to the negative control⁴².

CONCLUSION

The benefits that are achieved during these studies are undeniable, but ethical principles must be followed in using the number of animals tested and expected results. The use of animals for experimental purposes is of paramount importance, and it is up to the researcher to know how to choose the best tests that support the hypothesis towards the obtention of molecules with analgesic or anti-inflammatory potential.

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