

Original Article:

Isobolographic Antinociception of Nonsteroidal Anti-inflammatory Drugs in Rodent Formalin Orofacial Pain



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Article info:

Received: 21 Oct 2019

Accepted: 09 May 2019

Keywords:

Nonsteroidal anti-inflammatory drugs,
Orofacial pain,
Isobolographic analysis,
Synergism

ABSTRACT

Background: Diverse studies suggest that non-steroidal anti-inflammatory drugs (NSAIDs) induce antinociception through the inhibition of cyclooxygenases.

Objectives: This study evaluated the effect of NSAIDs in inducing antinociception either alone or in combination in mice formalin orofacial pain.

Methods: Male mice were injected intraperitoneally with dexibuprofen, dexketoprofen, diclofenac meloxicam, metamizole and piroxicam. Then from a dose-response curve the ED50 (dose that produce 50% of maximum effect) was obtained from each drug.

Results: The administration of NSAIDs produced a dose-dependent antinociception in both phases of the assay with different potency. Then, combinations of the cited NSAIDs were tested and analyzed by isobolographic analysis. The results demonstrate that the nocifensive response induced when dexketoprofen (DEX), the dextrorotatory enantiomer of the S (+) configuration of ketoprofen, was combined with piroxicam, diclofenac, dexibuprofen, metamizole, and meloxicam, was synergistic, either in Phase I or Phase II of the formalin orofacial mice assay.

Conclusion: The data demonstrated that the NSAIDs administered alone or in combination produce antinociception. These effects need to be explained by other mechanisms of action of NSAIDs other than the simple inhibition of COXs. The findings may be relevant for the relief of acute or chronic pain such as migraine, post-herpetic neuralgia and tooth pain.

Citation Noriega V, Sierralta F, Aranda N, Sotomayor-Zárate R, Poblete P, Prieto J C, Miranda HF. Isobolographic Antinociception of Nonsteroidal Anti-inflammatory Drugs in Rodent Formalin Orofacial Pain Pharmaceutical and Biomedical Research. 2020; 6(3):205-212. <http://dx.doi.org/10.18502/pbr.v6i3.4647>

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1. Introduction

Pain is a multidimensional experience, and animal tests based on the use of short/phasic and longer/tonic duration stimuli have been documented. To examine tonic pain models, the chemical stimulus has been recognized as the most appropriate tool for the study of acute and tonic pain states, and among them, the orofacial pain. This type of pain is related to common acute or chronic types of pain, such as migraine pain, post-herpetic neuralgia, and tooth pain [1].

To study orofacial pain in rodents, several substances have been used, including complete Freund's adjuvant, carrageenan, capsaicin, and formalin. The pain induced by the last substance has been considered as a short-term inflammatory pain model because it injures the tissue, activates nociceptors as well as trigeminal and spinal nociceptive neurons, and produces a painful sensation in humans. The administration of formalin produces a biphasic response, composed of an initial phase within the first minutes of post-administration (phase I), followed by an inactive period and a second phase (phase II) of around 20 minutes. Phase I is due to the direct chemical stimulation of nociceptive nerve endings with the release of substance P. Phase II involves both inflammatory mechanisms and central sensitization within the dorsal horn [1-3].

Orofacial pain is a painful syndrome associated with peripheral or central neural pathologies. Because of these features, the use of several drugs to combat this type of pain has been reported. These drugs include opioids such as tapentadol, tramadol, codeine, morphine, and fentanyl and non-steroidal anti-inflammatory drugs (NSAIDs) such as ketorolac, parecoxib, meloxicam, and dexketoprofen [4-10].

NSAIDs are commonly used in different types of pain and exhibit antipyretic, anti-inflammatory, and analgesic properties due to the inhibition of prostaglandin biosynthesis using cyclooxygenase enzymes. NSAIDs use other mechanisms such as alterations in interleukin release in microglial activity, as well as the activation of endogenous opioids and the like [11, 12].

Despite the variety of available antinociceptive drugs, the management of orofacial pain has been less researched compared to other types of pain, which in part seems to be insufficient due to the limited models of experimental assays. Besides, the difficulty of obtain-

ing effective analgesia with a single drug suggests that a drug combination (multimodal analgesia) should be used to treat pain to obtain synergistic therapeutic effects, reduces dose and toxicity, and minimizes drug resistance. Multimodal analgesia has been successfully tested in different types of pain, both acute and chronic ones [13-19].

The relevance or importance of testing multi-drugs in different algometric tests is associated with their marked differences in potency and efficacy of the doses used in relation to the nociceptive stimulus. Despite the variety of existing antinociceptive drugs, the management of orofacial pain has been less researched compared to other types of pain, which in part, seems to be due to the limited models of experimental assays. Furthermore, the difficulty of obtaining effective analgesia with the use of a single drug suggests that the use of several drugs should be used to treat pain to obtain synergistic therapeutic effects, reduce dose and toxicity and minimize drug resistance.

Therefore, the importance of the present study was to evaluate at the preclinical level, the type of multimodal analgesia between dexketoprofen (DEX) and either piroxicam, or diclofenac, or dexibuprofen, or metamizole, or meloxicam using the orofacial test of mice. The interaction was investigated by isobolographic analysis.

2. Materials and Methods

2.1. Study animals

Several male CF-1 mice (28-30 g) were collected and housed on a 12-hour light-dark cycle at 22±1°C with free access to food and water. The experimental procedures were approved by the Animal Care and Use Committee at the Faculty of Medicine, University of Chile (Protocol CBA 0410). The mice were accustomed to the laboratory for at least one hour before testing, used only once during the protocol, and euthanized by an overdose of anesthetic (60 mg/kg of pentobarbital) immediately after the algometric test. The number of animals was kept at a minimum, compatible with the consistent effects of the drug treatment. All the observers were blinded to the protocol of this study.

2.2. Measurement of antinociception

Analgesic activity was assessed by the orofacial formalin test, as previously described [10]. The orofacial formalin-induced responses displayed two distinct phases (separated by a period of relative inactivity) with an early, short-lasting response (zero to five minutes, phase

I) and a continuous, prolonged response (20-30 minutes, phase II). During the test, the mice were randomly assigned to different groups (six to eight per group), and 20 µL of 2% formalin solution was injected into the upper lips, right next to their noses, using a 27-gauge needle attached to a 50-µL Hamilton syringe. Each mouse was immediately returned to a Plexiglas observation chamber. Phase I corresponded to a 5-min period starting immediately after the formalin injection and represents tonic acute pain due to peripheral nociceptors sensitization. Phase II was recorded as a 10-min period starting 20 minutes after the formalin injection and represents inflammatory pain. The nociceptive score was determined for each phase by measuring the time the animals spent grooming the injected area.

Drugs or saline was administered to animals 30 min before formalin injection, a time at which preliminary experiments showed the occurrence of the maximum effect. Total grooming time in each period was converted to a percentage of maximum possible effect (%MPE) as follows:

$$\%MPE = 100 - \left(\frac{\text{post-drug grooming time} - \text{control grooming time}}{\text{control grooming time}} \right) \times 100$$

The dose that produced 50% of MPE (ED50) was calculated from the linear regression analysis of dose-response curves obtained by plotting log doses versus %MPE.

2.3. Analysis of drug interactions

The pharmacological interaction between DEX with either piroxicam (PIRO), or diclofenac (DICLO), or dexibuprofen (DEXIBU), or metamizole (META), or meloxicam (MELO), using the orofacial test of mice, was evaluated by an isobolographic analysis in agreement with the method previously described [20]. In brief, it was plotted on the x and y axes of the ED50 values of each drug alone. Then the line connecting the x and y

axes is the theoretical additive line or isobole. The point of ED50 of the combination was plotted; if the point of ED50 experimental falls above the isobole, synergy is present. The point fixed in the isobole is the theoretical additive point. Also, the magnitude of the interaction (I.I.) was calculated using the following equation:

$$I.I. = \frac{\text{Experimental ED50}}{\text{Theoretical ED50}}$$

If the value is close to 1, the interaction is additive; values below are an indication of a synergistic interaction [20].

2.3. Experimental design

To determine the antinociceptive potency of intraperitoneally (IP) NSAIDs, dose-response curves produced by 1, 3, 10, and 30 mg/kg for MELO, DICLO or DEX-IBU and 3, 10, 30, and 100 mg/kg in the case of DEX, PIRO, META were obtained. In the orofacial assay, six animals were used at least, for every four doses. The doses of NSAIDs must not induce changes in the motor activity of mice.

2.4. Drugs

Drugs were freshly dissolved in a sterile physiological salt solution of 10 mL/kg, for IP administration. Dexketoprofen was provided by Menarini, Spain, meloxicam, and metamizole by Saval Laboratories Chile, piroxicam by Pfizer Chile, diclofenac by Novartis Chile S.A., and dexibuprofen by Labomed Chile.

2.5. Statistical analysis

Results are presented as means ± SEM or as ED50 with 95% confidence limits (95% CL). The statistical difference between NSAIDs was assessed by 1-way ANOVA, followed by Turkey post hoc test, and P values less than 0.05 (P<0.05) were considered statistically significant.

Table 1. ED50 values with SEM in mg/kg, via i.p. for the antinociceptive effect of NSAIDs in the orofacial test of mice

NSAIDs	ED50 (mg/kg)	
	Phase I	Phase II
Meloxicam	8.04 ± 1.02	7.47 ± 1.50
Dexibuprofen	9.80 ± 1.08	16.89 ± 2.83
Diclofenac	13.54 ± 2.82	31.23 ± 5.65
Dexketoprofen	15.30 ± 2.51	53.68 ± 6.10
Piroxicam	33.56 ± 3.21	42.21 ± 6.99
Metamizol	36.56 ± 6.59	18.25 ± 3.10

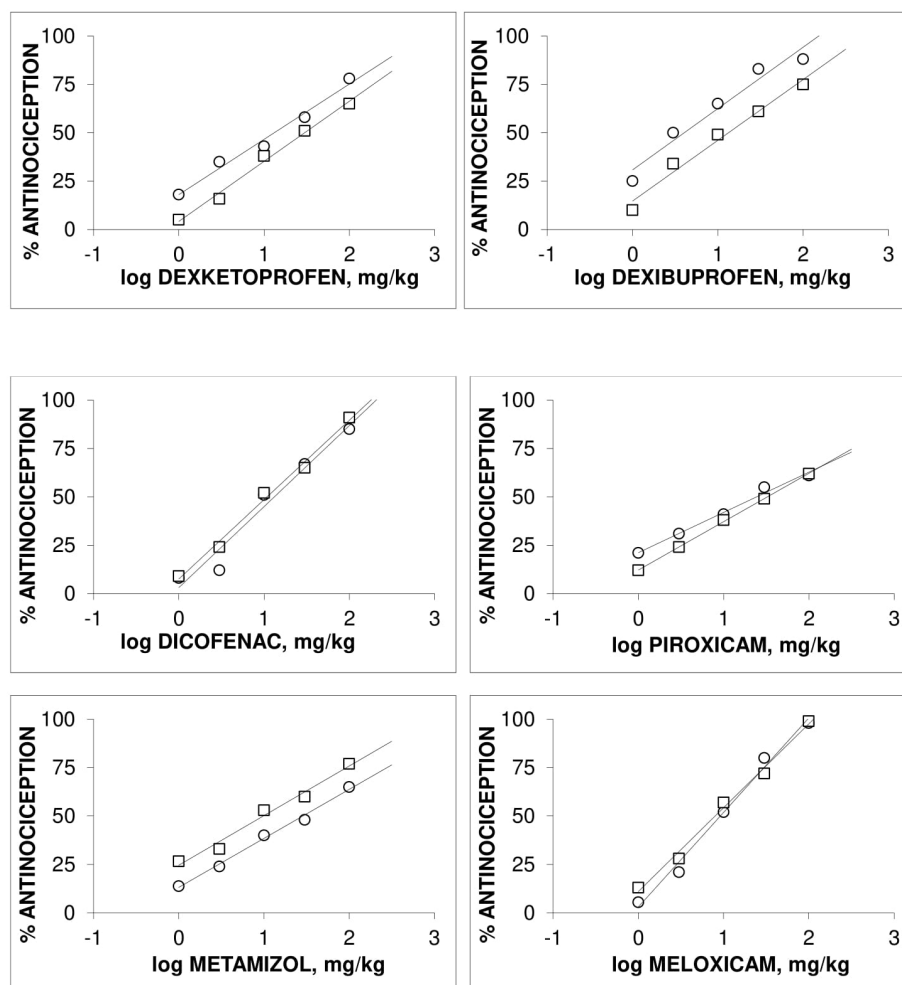


Figure 1. Dose-response curves for the antinociceptive activities in the formalin orofacial test

Test on mice, induced by the intraperitoneal administration of NSAIDs in phase I (O) and phase II (□). Each point is the mean response of six to eight animals.

Statistical analyses were carried out in Pharm Tools Pro, version 1.27 (McCary Group Inc., PA, USA).

3. Results

Antinociception by NSAIDs in mice formalin orofacial test

The IP administration of DEX, PIRO OR META at doses of 3, 10, 30, and 100 mg/kg and 1, 3, 10, and 30 mg/kg for MELO, DICLO, or DEXIBU displayed dose-dependent antinociception in the phase I and phase II of this test, with different potencies, as seen in the ED50 values shown in Table 1 and in Figure 1. Also, the findings demonstrate that MELO, DEXIBU, and DICLO exhibit greater power in phase I, but MELO, DEXIBU, and META, in phase II.

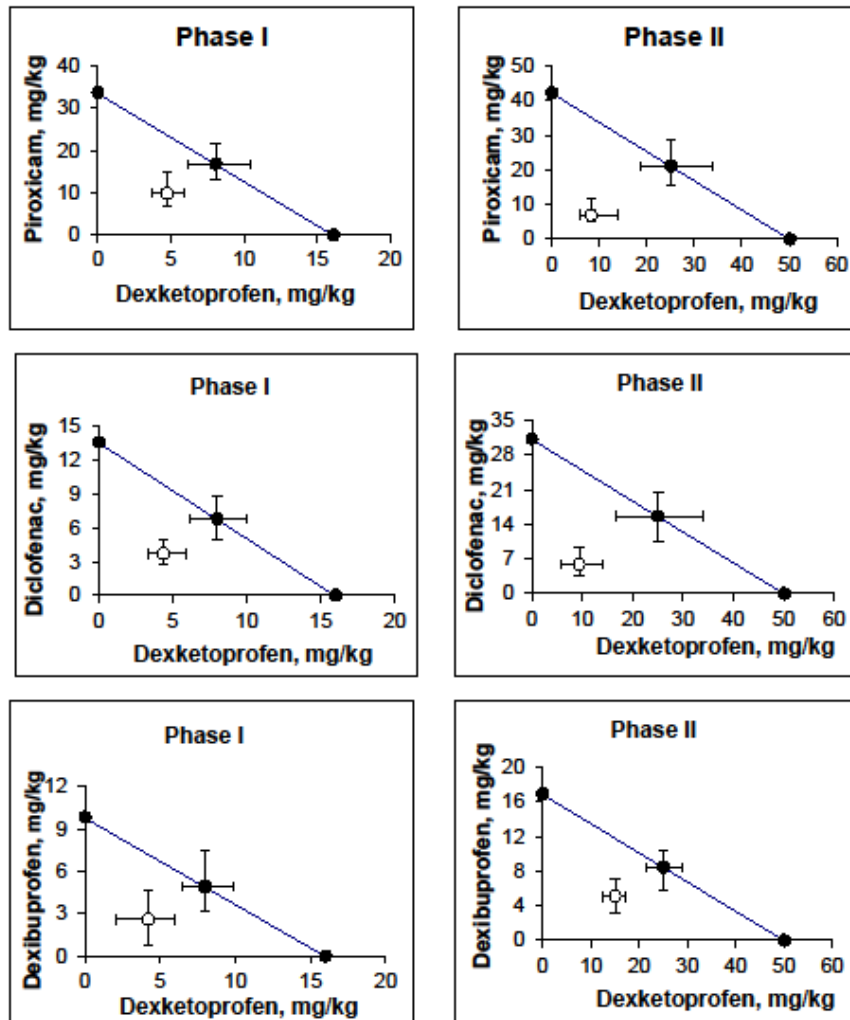
Isobolographic Analysis of NSAIDs in Combinations

The analysis of the results obtained by the co-administration of DEX with the different NSAIDs of this study were evaluated using the ratio 1:1 of their respective ED50 by isobolographic analysis. In the mice orofacial assay, the nocifensive response induced when DEX was combined with either DICLO, or META, or MELO, or DEXIBU or PIRO was synergistic, either in phase I or phase II. The interaction index (I.I.) obtained for all the combinations, in both phases, was lower than 1. All these results are shown in Table 2 and Figures 2 and 3.

4. Discussion

The results of the present study demonstrated that the intraperitoneal administration of DEX, piroxicam, di-

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Figure 2. Isobolograms for the intraperitoneal administration of the combination of DEX and piroxicam, diclofenac and dexibuprofen in the formalin orofacial test

Filled circles (●) are the theoretical ED₅₀s with 95% CL and open circles (○) are the experimental ED₅₀ with 95% CL.

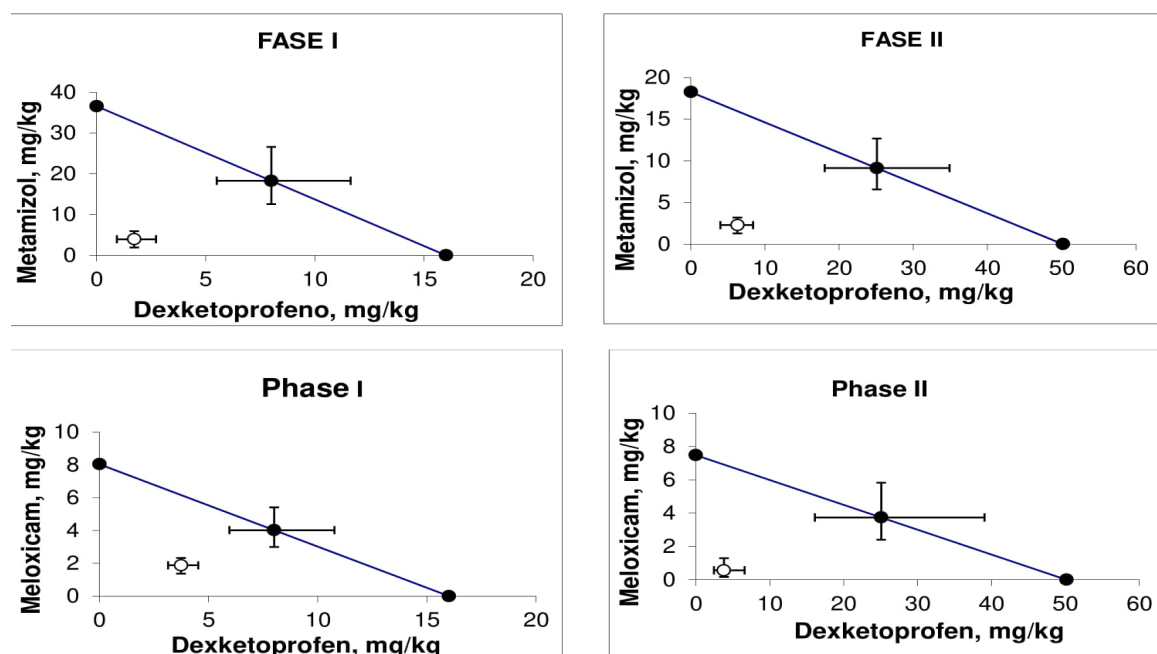
diclofenac, dexibuprofen, metamizole, and meloxicam produce dose-dependent antinociception with dissimilar relative potencies in different types of nociceptive stimulus in experimental pain of mice [4-10].

The findings obtained by the administration of each of the NSAIDs, in the orofacial test, according to the NSAIDs-COXs mechanism of action, indicate that the

Table 2. Interaction index (I.I.) of the i.p. combination of dexketoprofen (DEX) with NSAIDs in both phases of the orofacial test of mice.

Combination	Phase I	Phase II
Diclofenac/DEX	0.19	0.37
Metamizol/DEX	0.22	0.25
Meloxicam/DEX	0.47	0.15
Dexibuprofen/DEX	0.52	0.60
Piroxicam/DEX	0.58	0.33

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Figure 3. Isobolograms for the intraperitoneal administration of the combination of DEX and metamizole and meloxicam in the formalin orofacial test

Filled circles (●) are the theoretical ED'50s with 95% CL and open circles (○) are the experimental ED50 with 95% CL.

inhibition of COX-2, in both phases of the test, is superior to the inhibition of COX-1 and COX-3.

Regarding the antinociceptive activity of the combinations of DEX with either DICLO, or META, or MELO, or DEXIBU, or PIRO, the isobolographic analysis revealed a synergic interaction in all combinations, in phase I and phase II. The differences in the magnitude of the observed interactions between the different NSAIDs may be related to multiple events and expressed by the interaction index and could be related to their COXs selectivity, the potency of COXs inhibition, pharmacokinetic properties, or other mechanisms of action. The interaction could occur at one or more levels of cell function, i.e., receptor, ion channels, second messengers, protein kinases, or others. These events are dependent on the local concentration of drugs and the nociceptive stimulus [21].

The pain modulation is mediated through diverse mechanisms, so the synergistic interaction induced by combinations of NSAIDs cannot be explained only by a simple mechanism of the inhibition exerted by COXs in the concentration of prostaglandin. However, the existence of other mechanisms of action could contribute to the explanation of the present synergism. Furthermore, a wealth of evidence demonstrates the effects of NSAIDs beyond the action on COXs, which include

their interaction with cholinergic, adrenergic, serotonergic and cannabinoid, and nitric oxide systems. Besides, molecular biology suggests multiple pathways that may be related to the analgesic and anti-inflammatory effects of NSAIDs [22].

The existence of other mechanisms of action that have been assigned to NSAIDs could contribute to the synergism detected in this study, including the induction of downregulation of L-selectin (diclofenac), inhibition of NOS activity by DEX, inhibition of nuclear factor kappa B (dexibuprofen, [DEX]), inhibition of β_2 integrin activation (piroxicam, meloxicam), inhibition of very late activation, VLA-4 (diclofenac), reduction in pronociceptive cytokines: IL-1 β , XCL1, and CCL2 (metamizole) [23, 24].

To explain the synergy obtained in the present study, it has been used the different mechanisms that have been informed for NSAIDs; however, it has been proven that for the determination of synergism it is not necessary to know the mechanisms of action of drugs, since it is based on the law of mass action which is independent of the mechanism. On the other hand, some drugs have more than one mechanism of action, so it would be difficult to determine their exact proportion and mechanism of actions in the synergy [25].

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5. Conclusions

The present results support that intraperitoneal administration of NSAIDs alone induces antinociception and in combination produces synergism, in the formalin orofacial model. Also, this effect appears to be mediated by additional mechanisms of action besides COXs inhibition.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article. Participants were informed about the purpose, implementations stage, confidentiality of information, free from the study when they wished and the results were available to them. The study was approved by the Animal Care and Use Committee at the Faculty of Medicine, University of Chile (Protocol CBA 852/FMUCH/2018).

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors' contributions

All authors equally contributed in preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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