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Isobolographic analysis of the antinociceptive interactions of clonidine with nonsteroidal anti-inflammatory drugs

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Abstract

The present study was undertaken to characterize the interactions between nonsteroidal anti-inflammatory drugs and the α_2-adrenoceptor agonist clonidine in an acute nociceptive test. The writhing test was selected as a model of acute visceral pain. Isobolograms were constructed to assess the interactions of clonidine and each nonsteroidal anti-inflammatory drugs, when coadministered intraperitoneally and intrathecally (i.t.). The simultaneous intraperitoneal administration of fixed ratios of ED_{50} fractions of all nonsteroidal anti-inflammatory drugs (naproxen, piroxicam, paracetamol, dipyrone or metamizol and nimesulide) combined with clonidine resulted in synergistic interactions. The same combinations administered intrathecally were additive. The synergistic interactions between systemic nonsteroidal anti-inflammatory drugs and clonidine may involve supraspinal mechanisms.

Keywords: Antinociception; Clonidine; NSAIDs; Isobolograms; Synergy

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of a variety of pain and inflammatory disorders. The molecular target of NSAIDs is the cyclooxygenase enzyme (COX). Some NSAIDs such as ketoprofen, indomethacin, aspirin, naproxen and ibuprofen are selective inhibitors of constitutive COX-1, while others, such as meloxicam, nimesulide, celecoxib and rofecoxib are mainly inhibitors of inducible COX-2 [1,2]. However, it is clear that NSAIDs exert their analgesic effect not only through peripheral inhibition of prostaglandin biosynthesis, but also through a variety of other peripheral and central mechanisms [1–7].

Many studies have demonstrated that antinociception is partially mediated via α_2-adrenoceptors at both spinal and supraspinal sites [8]. Clonidine is an agonist of α_2-adrenoceptors that has been employed both experimentally and clinically in the management of pain, either alone or in combination with opioids [9,10]. The antinociceptive activity of clonidine is observed after systemic or intrathecal (i.t.) administration and several mechanisms have been postulated for this activity, including interactions mediated by α_2-adrenoceptors at peripheral, spinal and supraspinal sites [11], supraspinal GABA receptors [12], histamine receptors [13], cholinergic mechanisms of analgesia [14], the ascending and descending adrenergic pathways [4,6], the L-arginine–NO system [15] or the opioid system [16]. Clonidine developes antinociceptive synergy with opioids [10,16], with serotonergic agonists [17] and with the NSAID ketorolac [18]. Nevertheless, the antinociceptive activity of clonidine was found to be only additive with the cholinergic receptor agonist carbachol, the indirect acting cholinergic receptor agonist physostigmine, the GABA receptor agonist midazolam [12] and neostigmine [5,9]. Midazolam and clonidine are reported to be synergistic [19]. The synergistic effect should be clinically useful, since both clonidine and NSAIDS have a place in the treatment of visceral and post-operative pain [9,20].

The purpose of the present study was to determine, using a chemical viscero-somatic noxious stimulus, if some modulatory interactions can be demonstrated between the antinociceptive effect of NSAIDs and the selective α_2-adrenoceptor agonist clonidine, with intrathecal and systemic administrations.

2. Materials and methods

CF-1 male and female mice of 35–40 days of age, weighing 29 ± 1.5 g, were used. The animals were acclimatized

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to the laboratory environment for at least 2 h before used. Ethical standard guidelines were followed as previously described [6] and were approved by the local ethical committee. Each animal was used only once and received only one dose of the drugs tested. All drugs were freshly prepared by dissolving in normal saline or in a slightly hyperbaric solution of glucose (6%) and doses were calculated on the basis of the drug salts. All observations during the assay were performed by the authors in a randomized and blinded manner. Evaluation of antinociceptive activity was accomplished as previously reported [5]. Briefly, i.p. administration was done by injecting the total dose in a constant volume of 10 ml kg^{-1}, 30 min before the algesiometric test. For intrathecal administration, the Hylden and Wilcox technique [21] was used, and the drugs were injected 15 min before the algesiometric test, in a constant volume of 5 μl to 30 g mice, dissolved in a slightly hyperbaric solution of glucose (6%) to limit rapid diffusion of the drug to higher levels of the spinal cord. The times of drug administration before the algesiometric test (30 min for i.p. and 15 min for i.t.) were found in previous experiments to be near the time to reach the maximum analgesic effect [5]. Control animals (saline or 6% glucose) were run interspersed concurrently with the drug-treated animals (at least two controls per group), which prevented all the controls being run on a single group of mice at one time during the course of the investigation.

2.1. Algesiometric test (writhing test)

The writhing test was selected as a model of acute visceral pain, because it can be a model of clinical relevant intestinal pain in humans [22]. Mice were injected i.p. with 10 ml kg^{-1} of 0.6% acetic acid and the number of writhes was counted during a 5 min period, starting 5 min after the administration of acetic acid solution. A writhe was defined as a contraction of the abdominal muscles accompanied by an elongation of the body and extension of the hindlimbs. Dose-response curves, determined near the time of peak effect, were constructed in order to assess the antinociceptive action of the different NSAIDs and clonidine given i.p. or i.t. Eight animals were used at each of at least four doses to determine a dose-response curve. The dose that produced 50% of antinociception (ED_{50}: 50% reduction of control writhes) was calculated using standard linear regression analysis of the dose-response curve and the equation of the straight line. Antinociceptive activity was expressed as per cent inhibition of the usual number of writhes observed in control animals injected i.p. with saline (24.3 ± 1.0, $n = 52$) or i.t. with glucose (23.9 ± 0.5, $n = 50$).

2.2. Isobolographic analysis

The interaction of clonidine with the antinociceptive effect of NSAIDs was evaluated by simultaneous administration of fixed ratios of clonidine with each NSAID, and performing an isobolographic analysis for the different combinations, as described by Tallarida et al. [23,24]. The isobologram was constructed by connecting the ED_{50} of the corresponding NSAID plotted on the abscissa with the ED_{50} of clonidine plotted on the ordinate to obtain the additivity line. For each drug mixture, the ED_{50} and its associated 95% confidence intervals were determined by linear regression analysis of the log dose-response curve (eight animals at each of at least four doses) and compared by a *t*-test to a theoretical additive ED_{50} obtained from the calculation: $ED_{50\,add} = ED_{50\,NSAID}/(P1 + R \times P2)$, where R is the potency ratio of the NSAID alone to clonidine alone, $P1$ is the proportion of NSAID and $P2$ is the proportion of clonidine in the total mixture [5,6]. In the present study, fixed-ratio proportions were selected by first combining the ED_{50} and then constructing a dose-response curve in which ED_{50} fractions (1/2, 1/4, 1/8 and 1/16) of clonidine/NSAID combinations were administered. In the equation above, ED_{50add} is the total dose and the variance of ED_{50add} was calculated from the fraction of the ED_{50} (i.e. 0.5) in the combination as: $\text{Var}\ ED_{50\,add} = (0.5)^2 \times \text{Var}\ ED_{50\,NSAID} + (0.5)^2 \times \text{Var}\ ED_{50\,CLONIDINE}$ [5,6]. From these variances, confidence intervals were calculated and resolved according to the ratio of the individual drugs in the combination. Supra-additivity or synergistic effect is defined as the effect of a drug combination that is higher and statistically different (ED_{50} significantly lower) than the theoretical calculated equieffect of a drug combination with the same proportions. When the drug combination gives an experimental ED_{50} not statistically different from the theoretically calculated ED_{50}, the combination has an additive effect and additivity means that each constituent contributes with its own potency and the less potent drug is acting as though it is merely a diluted form of the other [24].

2.3. Drugs

All drugs used were dissolved in saline solution when they were administered i.p. or in a slightly hyperbaric solution of glucose (6%) to limit diffusion, in i.t. administration. Nimesulide was dissolved in 5 mM dimethylsulfoxide; the vehicles were devoid of antinociceptive activity when tested i.p or i.t. The drugs, provided by local subsidiaries of international pharmaceutical companies, were: naproxen, piroxicam, paracetamol, dipyrone or metamizol and nimesulide. Clonidine hydrochloride was purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.4. Statistical analysis

Results are presented as mean values ± S.E.M. or ED_{50} values with 95% confidence interval (95% C.I). Statistical significance for the Student's *t*-test used to compare theoretical and experimental points in the isobolograms was accepted at $P < 0.05$.

Table 1
ED_{50} values with 95% confidence intervals (CI) for the antinociceptive effect of NSAIDs and clonidine administered intraperitoneally or intrathecally

Drugs	ED_{50} (95%CI) (mg kg^{-1})	
	Intraperitoneal[a]	Intrathecal
Piroxicam	8.5 (6.4–11.2)	0.5 (0.4–0.6)
Metamizol	28.5 (21.8–37.6)	0.8 (0.4–1.6)
Paracetamol	221.0 (141–346.7)	9.3 (7.9–10.8)
Nimesulide	7.6 (5.8–9.8)	0.29 (0.2–0.5)
Naproxen	46.4 (35.7–60.5)	6.6 (4.7–9.1)
Clonidine	0.00041 (0.00034–0.00050)	0.000036 (0.000031–0.000042)

[a] $P < 0.05$ between intraperitoneal and intrathecal values.

3. Results

3.1. Antinociceptive effects of NSAIDs and clonidine in the writhing test

The systemic (i.p.) or inthathecal administration of the different NSAIDs and clonidine produced dose-dependent antinociception in the algesiometric assay of acetic acid-induced writhes. The dose-response curves were characterized by equal efficacy and the corresponding ED_{50}, with 95% confidence intervals appear in Table 1. With the antinociceptive doses used, no significant behavioral or motor dysfunction was observed in the animals. The vehicles (saline, glucose and dimethylsulfoxide) were devoid of antinociceptive activity when tested i.p or i.t.

3.2. Interactions of NSAIDs and clonidine

The antinociceptive activity induced by the coadministration of fixed ratios of ED_{50} fractions of NSAIDs and clonidine was examined by isobolographic analysis after i.p. or i.t. administration. The ED_{50} values for the combinations and their 95% confidence intervals are shown in Tables 2 and 3. Fig. 1 shows an example of the log dose-response

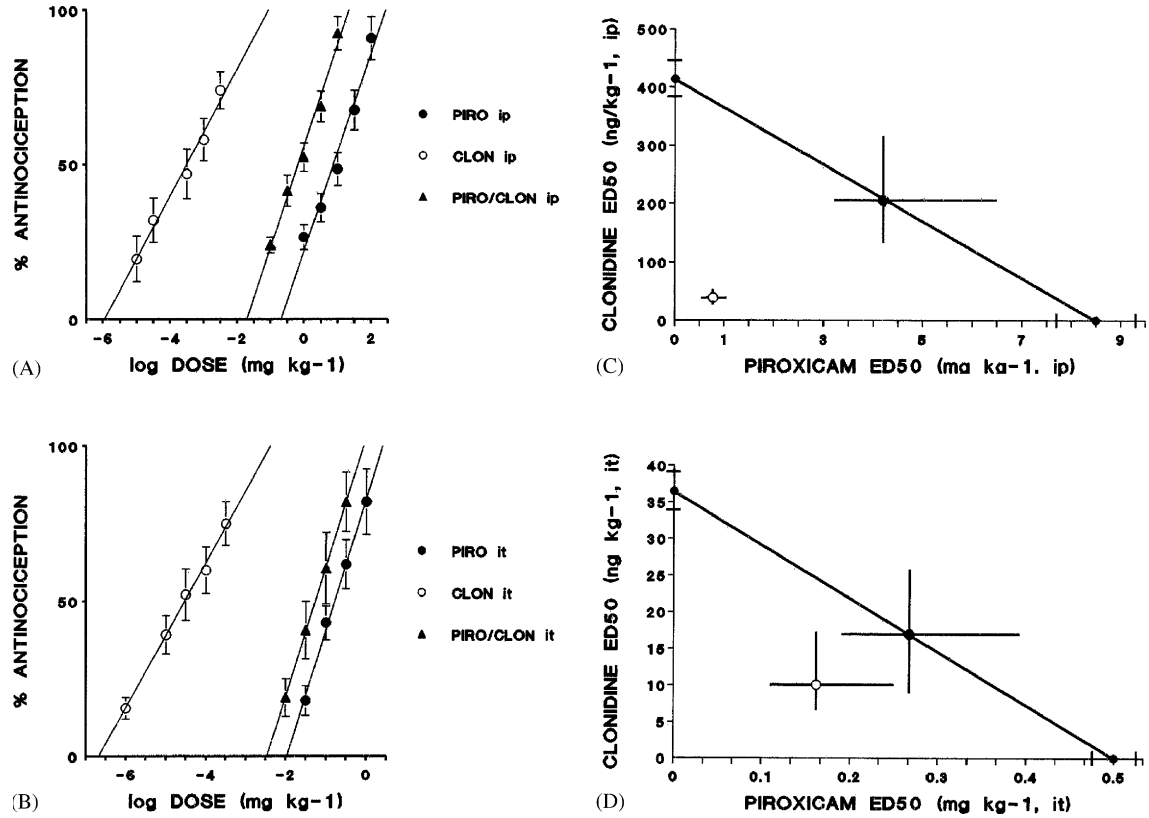

Fig. 1. Dose-response curves for the administration of piroxicam (PIRO), clonidine (CLON) and the simultaneous administration of PIRO/CLON (Panel A: intraperitoneal, i.p.; Panel B: intrathecal, i.t.). Each point is the mean ± S.E.M. of eight animals. Isobologram for the simultaneous administration of piroxicam and clonidine (Panel C: intraperitoneal, i.p.; Panel D: intrathecal, i.t.; $n = 8$ animals). Horizontal lines in the abscissa and the ordinate represent the S.E.M. of the corresponding ED_{50}. Filled circles correspond to the theoretical ED_{50} with 95% confidence intervals and open circles correspond to the experimental ED_{50} with 95% confidence intervals. Ordinates and abscissas of isobolograms are on different scales.

Table 2
Theoretical and experimental ED_{50} values with 95% confidence intervals (CI) for combinations of NSAIDs and clonidine administered intraperitoneally

Drugs	ED_{50} (95%CI) (mg kg^{-1})	
	Theoretical	Experimental
Piroxicam/clonidine	4.25 (2.70–7.31)	0.86[a] (0.69–1.07)
Metamizol/clonidine	14.25 (8.89–22.82)	8.77[a] (6.64–11.58)
Paracetamol/clonidine	110.50 (64.76–188.55)	38.96[a] (30.35–50.03)
Nimesulide/clonidine	3.79 (2.75–6.52)	1.76[a] (1.42–2.65)
Naproxen/clonidine	23.20 (11.70–40.88)	10.54[a] (6.79–16.37)

The ratio between the NSAID and clonidine (1:1) corresponds to the ratio of the respective ED_{50} (see Section 2).
[a] $P < 0.05$ between theoretical and experimental values.

Table 3
Theoretical and experimental ED_{50} values with 95% confidence intervals (CI) for combinations of NSAIDs and clonidine administered intrathecally

Drugs	ED_{50} (95%CI) (mg kg^{-1})	
	Theoretical	Experimental
Piroxicam/clonidine	0.27 (0.18–0.40)	0.17 (0.11–0.24)
Metamizol/clonidine	0.40 (0.19–0.84)	0.27 (0.17–0.40)
Paracetamo/clonidine	4.65 (3.32–6.48)	2.79 (1.95–3.99)
Nimesulide/clonidine	0.15 (0.11–0.22)	0.10 (0.07–0.14)
Naproxen/clonidine	3.46 (1.97–6.09)	2.45 (2.12–2.84)

The ratio between the NSAID and clonidine (1:1) corresponds to the ratio of the respective ED_{50} (see Section 2).

curves obtained, determined for piroxicam and for the combination piroxicam/clonidine administered i.p. (Fig. 1A) and i.t. (Fig. 1B), from where the experimental ED_{50} for the combinations were calculated. The isobolographic analysis for the combination piroxicam/clonidine administered i.p., resulted in a synergistic interaction (Fig. 1C), however, the same combination administered i.t. was only additive (Fig. 1D), since the experimental value was not statistically different from the calculated theoretical value. The concurrent i.p. administration of clonidine with either metamizol (Fig. 2A), nimesulide (Fig. 3A), paracetamol (Fig. 2C) or naproxen (Fig. 3C) resulted in synergistic interactions. For all the combinations of clonidine and NSAIDs administered i.t., an additive interaction was obtained (Figs. 2 and 3; Table 3).

4. Discussion

It has been previously reported that systemic and/or intrathecal administration of either NSAIDs or clonidine produced antinociceptive activity in experimental animals [8,10] and in human volunteers [9], in several algesiometric assays. The results obtained in the present work are in agreement with these findings.

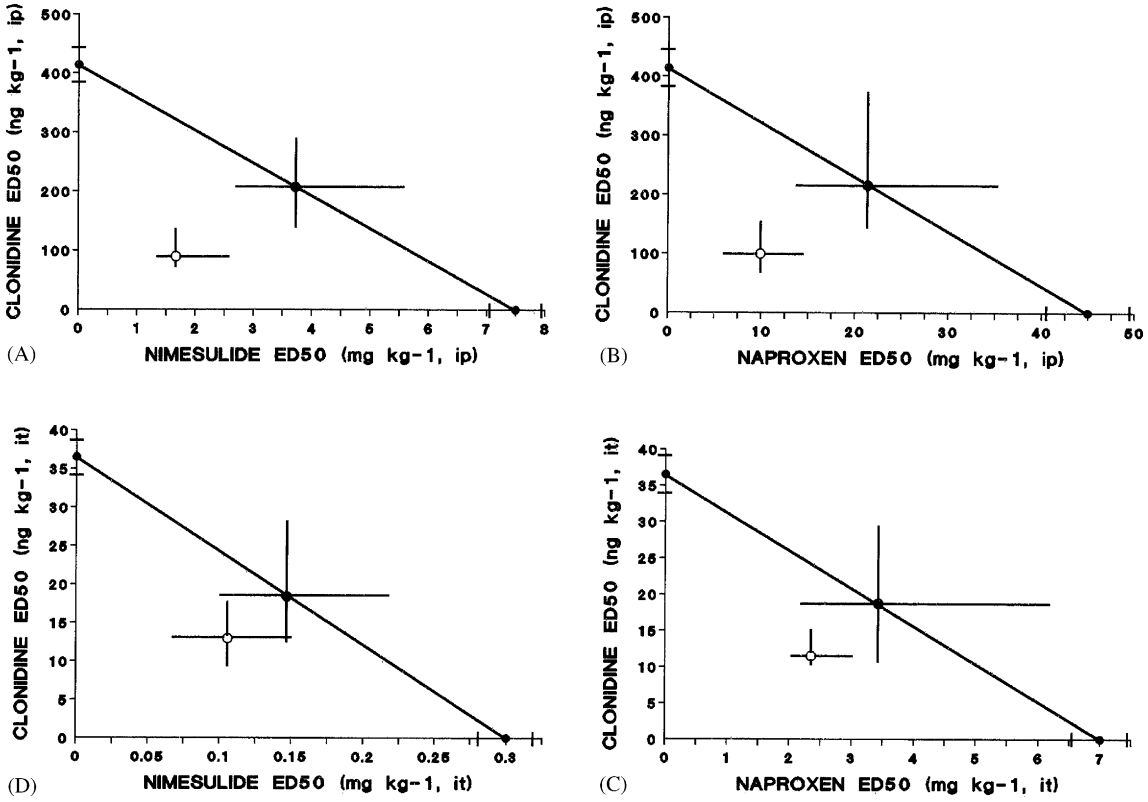

Fig. 2. Isobolograms for the antinociception induced by the coadministration of clonidine + nimesulide (Panel A: intraperitoneal, i.p.; Panel B: intrathecal, i.t.) and clonidine + naproxen (Panel C: intraperitoneal, i.p.; Panel D: intrathecal, i.t.). Symbols and scales as in Fig. 1.

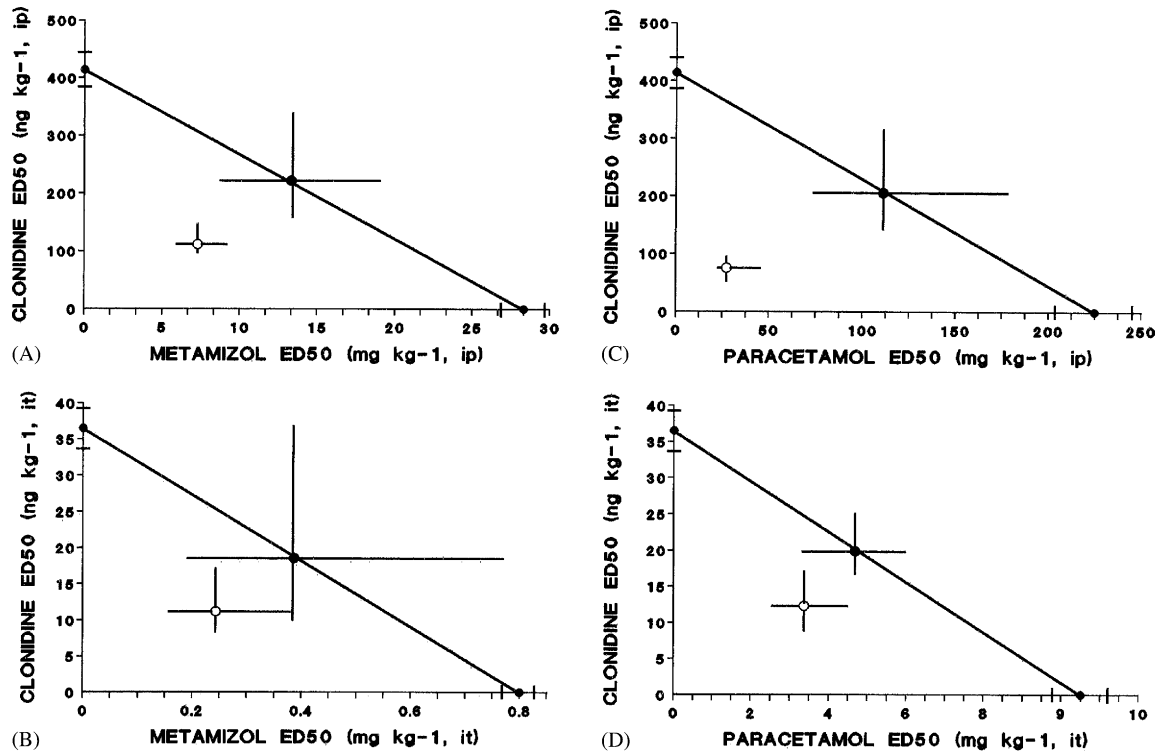

Fig. 3. Isobolograms for the antinociception induced by the coadministration of clonidine + metamizol (Panel A: intraperitoneal, i.p.; Panel B: intrathecal, i.t.) and clonidine + paracetamol (Panel C: intraperitoneal, i.p.; Panel D: intrathecal, i.t.). Symbols and scales as in Fig. 1.

A synergistic interaction between α_2-adrenoceptor agonists and opioids has been demonstrated and, since these drugs are significantly more potent after i.t. administration than after other routes, it was suggested that the most likely site of interaction is spinal [16]. Furthermore, the antinociceptive effect of spinal α_2-adrenoceptor agonists seems to be due to a direct action on dorsal horn neurons and not to the activation of descending inhibitory systems [25]. The different results between i.p. and i.t. administrations, are in agreement with previous works, in which synergistic antinociceptive interactions were obtained after systemic administration, but not after intrathecal delivery, since systemically administered drugs may presumably reach both supraspinal and spinal sites and activate descending inhibitory systems [4,6]. Synergistic interactions could be due to activation of different pathways of pain inhibition, and systemic administration can reach supraspinal sites and would predominantly stimulate descending inhibitory pathways, usually noradrenergic and serotonergic [11]. However, if the drugs are administered only intrathecally, one of these pathways would not be involved in this activation [10].

In the present work, the supra-additivity observed after the i.p. administration of clonidine and NSAIDs could be due to the fact that they act through different mechanisms, i.e. direct action on dorsal horn neurons for clonidine and activation of descending inhibitory mechanisms in addition to spinal COX-2 inhibition for NSAIDs [6,14,16]. Consequently, the systemic administration of the combination of NSAIDs and clonidine would produce synergistic interactions. The fact that adrenoceptor agonists interact with NSAIDs by synergistic mechanisms could be interpreted as an indication of the activation of different and complementary mechanisms of antinociception, since the activation of a common mechanism should presumably produce an additive interaction [4,10].

It has been reported that antinociception induced by α_2-adrenoceptor agonists is significantly influenced by interactions with other receptors (e.g. opioid, serotonergic, muscarinic) and can activate a variety of antinociceptive mechanisms, depending on the dose and the route of administration of the drugs [26]. The binding of α_2-adrenoceptor agonists was found also on non-primary afferent terminals and cell bodies, where increased K^+ conduction hyperpolarize the cell and reduce excitability [27]. According to single neurons electrophysiological recordings, nociceptive signals at supraspinal levels are more sensitive to antinociceptive effects of systemically administered α_2-adrenoceptor agonists than nociceptive signals at the spinal cord level [25]. On the other hand, α_2-adrenoceptor agonists spinally may inhibit the activity of the preganglionic sympathetic neurons, reducing sympathetic outflow [27] and, to the extent to which nociceptive transmission is driven by sympathetic input, the nociception would be diminished.

The findings of the present study show that when clonidine was coadministered systemically with naproxen, piroxicam, paracetamol, metamizol or nimesulide at sub-ED_{50}

doses, significant synergistic interactions were obtained, in agreement with the work of Jain using tizanidine, an α_2-adrenoceptor agonists, demonstrated a significant potentiation of antinociceptive effects of nimesulide, meloxicam and naproxen in the same algesiometric test [28]. In general, the association of clonidine with a lower dose of a COX non-specific NSAID might decrease significantly the side effect profile (i.e. GI ulcerations and bleeding) seen with non-specific NSAIDs [28].

In conclusion, these results demonstrate systemic synergistic interactions between NSAIDs and clonidine, which may be clinically interesting, because the use of combinations can reduce the total amount of any one drug required for antinociception.

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