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Synergism between NSAIDs in the orofacial formalin test in mice

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ABSTRACT

Opioids and non-steroidal anti-inflammatory drugs (NSAIDs) are used to relieve acute and chronic pain. The purpose of this study was to determine the degree of interaction between dexketoprofen and NSAID examples of COXs inhibitors using the isobolographic analysis in the formalin orofacial test in mice. The drugs, i.p., induced a dose-dependent antinociception with different potencies in both test phases. Combinations of dexketoprofen with naproxen, nimesulide, ibuprofen or paracetamol on the basis of the fixed ratio (1:1) of their ED_{50}'s values alone demonstrated synergism in both phases. This is important since the orofacial pain is a test not currently used in mice; the drugs are all analgesic for humans and phase II is representative of inflammatory pain. The synergism was: COX-3>COX-2>COX-1 inhibitors, this is particularly interesting since the inhibitor of COX-3, paracetamol, displayed a robust anti-inflammatory activity in an assay of acute and inflammatory pain that mimics inflammatory pain in humans.
In conclusion, the synergism of the dexketoprofen/NSAID combinations may improve this type of therapeutic profile, since with low doses of the components, side effects are not likely to occur, and they may be used in long-term treatments.

1. Introduction

Pain is a complex, multidimensional experience that has a particular expression in the oro-facial region since the face and mouth have a special biological, emotional, and psychological meaning for each individual. The face and mouth represent places in the body where the most common pains are felt. Many of the difficulties in the management of acute and chronic oro-facial pain conditions stem from a lack of recognition and understanding of oro-facial pain mechanisms.

The management of pain continues being a major challenge for medicine. Opioids and non-steroidal anti-inflammatory drugs (NSAIDs) are the main agents used to relieve acute and chronic pain.

NSAID action has been attributed to the inhibition of the prostanoid synthesis in the tissues, by inhibiting the cyclooxygenase (COX) enzyme. COX-1, a cyclooxygenase isoform is constitutively expressed in almost all cells, and naproxen is one of the examples of a COX-1 inhibitor. COX-2, another isoform, is a highly inducible enzyme by various stimuli and Nimesulide is an example of an NSAID inhibitor of this enzyme. In addition, Ibuprofen is a prototype of COX-1 and COX-2 inhibitor (Warner and Mitchell, 2004). Recently, a third isoform, COX-3, has been described by Chandrasekharan et al. (2002) that is a splice variant of COX-1 and is related to the action mechanism of paracetamol or acetaminophen (Vane, 2000).

It is well known that tissue injury causes the release of various inflammatory and pain mediators resulting in peripheral sensitization. The pain response mediators: ATP, acetylcholine and serotonin are released from damaged endothelial cells and platelets; prostaglandin E2 is synthesized by COX enzymes in damaged cells; bradykinin is released from damaged vessel plasma. The inflammatory response mediators include: histamine that is released from mast cells in response to Substance P and calcitonin gene-related peptide (CGRP) is released by primary afferent sensory fibres; additional mediators are released from blood cells, such as cytokines, complement factors C3a and C5a, serotonin, platelet-activating factor, neutrophile chemotactic factor, fibrinopeptides, leukotrienes, etc (Furst, 1999; Millan, 1999).

Several receptors and neurotransmitters are involved in the nociceptive system, some of them increasing and others inhibiting the pain sensation both peripherally and centrally. A hypothesis assumes that the simultaneous activation of different pain inhibiting pathways may be effective in pain therapy (Horvath and Kekesi, 2006). Thus, the co-administration of drugs that interfere with different systems may be an effective method to relieve pain. The combination of different NSAIDs activates both central and peripheral pain pathways to induce synergistic antinociception, and this interaction may allow lower doses of each drug combined and improve the safety profile, with lower side-effects (Desmeules et al., 2003).

It has been shown that the co-administration of the S(+) dextrorotatory enantiomer of the racemic ketoprofen, named dexketoprofen, is one of the most selective COX-1 inhibitors clinically available. In

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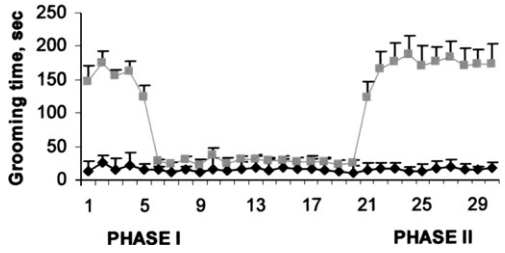

Fig. 1. Time course of the grooming activity of the oro-facial formalin test in mice. Formalin (■) and (◆) saline. Each point represents the mean with S.E.M. of at least 6 mice.

addition, dexketoprofen co-administered either with morphine or paracetamol, induced synergy in the acetic acid writhing test, the tail flick test and the formalin injection in the mice's hind paw (Miranda et al., 2007).

The purpose of this study was to determine the degree of interaction (i.e. additive or synergistic) between dexketoprofen and other NSAIDs examples of different Cox inhibitors as naproxen, nimesulide, ibuprofen, or paracetamol. The type of interaction was evaluated by means of the isobolographic analysis using the formalin oro-facial test in mice. This assay was selected since the face and mouth have a special biological, emotional, and psychological meaning for each individual. Furthermore, the face and mouth represent places in the body where most of the common pains occur.

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 in the laboratory environment for at least 2 h before use. Experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and experimental procedures were approved by the Institutional Animal Care and Use Committee at the Universidad de Chile, Santiago, Chile. 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 and administered intraperitoneally (i.p.). All observations during the assay were performed by the authors in a randomized and blind manner. Control animal saline was 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. Orofacial formalin test

The orofacial formalin-induced responses showed two distinct phases that were separated by a period of relative inactivity with an early short-lasting response (0–5 min, phase I) and a continuous prolonged response (20–30 min, phase II). To perform the test, mice were randomly assigned to different groups (6–8 per group) and 20 µL of 5% formalin solution was injected into the upper lip, right next to the nose with a 27-gauge needle attached to a 50 µL Hamilton syringe (Luccarini et al., 2006). The applied chemical stimulus (formalin) can be considered noxious since it produces tissue injury, activates Aδ and C nociceptors as well as trigeminal and spinal nociceptive neurons and produces a painful sensation in humans (Raboisson and Dallel, 2004). Each mouse was immediately returned to a Plexiglas observation chamber. The test shows two clear cut phases: Phase I corresponds to the 5 min period starting immediately after the formalin injection and represents a tonic acute pain due to peripheral nociceptor sensitization. Phase II was recorded as the 10 min period starting 20 min after the formalin injection and represents inflammatory pain. The nociceptive score was determined for each phase by measuring the total number of seconds that the animals spent grooming the injected area with the ipsilateral fore or hindpaw (Luccarini et al., 2006).

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

$$\%MPE = 100-(\text{post drug grooming time}/\text{control grooming time saline})\times 100.$$

The dose that produced 50% of MPE (ED_{50}) was calculated from the linear regression analysis of a dose–response curve obtained by plotting log doses versus % MPE.

2.2. Protocol

Dose–response curves for i.p. administration of dexketoprofen, ibuprofen, nimesulide, paracetamol or naproxen were obtained using at least six animals at each of at least four doses. A least-square linear regression analysis of the log dose–response curve allowed the calculation of the doses that produced 50% of antinociception when each drug was administered alone. ED_{50} was used in the orofacial formalin tests as the equieffective dose for isobolographic analysis because higher doses did not show increased effects without motor

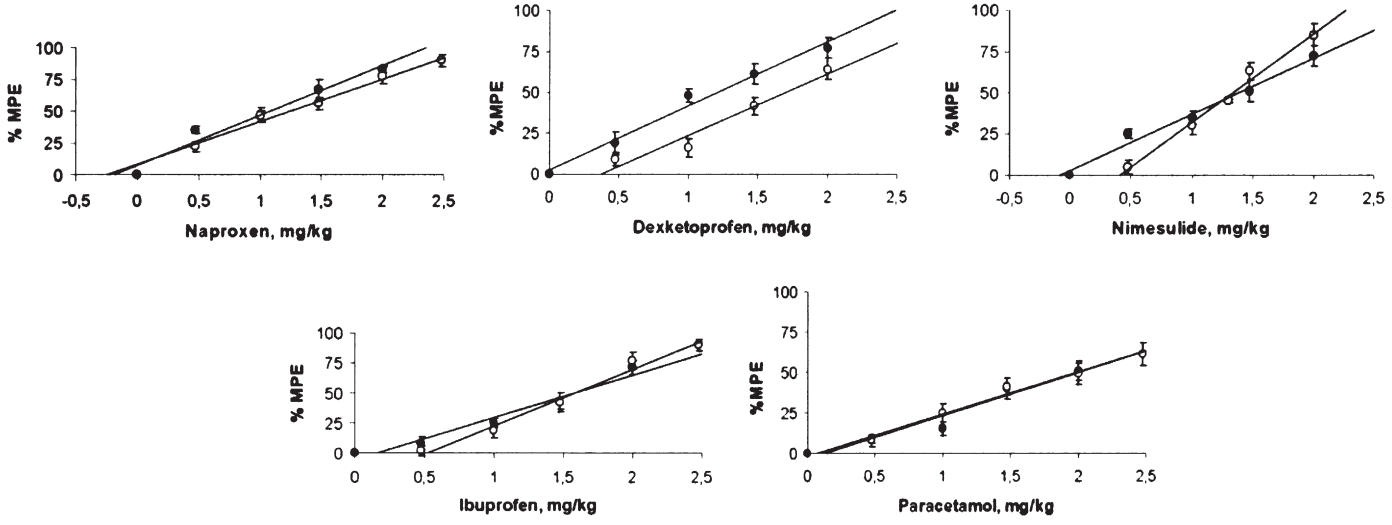

Fig. 2. Dose–response curves for the antinociceptive activity induced by intraperitoneal administration of naproxen, dexketoprofen, nimesulide, ibuprofen and paracetamol, in the orofacial formalin assay with mice. Each point is the mean±S.E.M. of 6–8 animals.

Table 1
ED_{50} values with S.E.M. for the antinociceptive effect of intraperitoneal administration of dexketoprofen, naproxen, nimesulide, ibuprofen and paracetamol in the phase I and the phase II of the orofacial formalin test of mice

Drugs	ED_{50} ± S.E.M. (mg/kg i.p.)	
	Phase I	Phase II
Dexketoprofen	16.00 ± 2.59	50.16 ± 8.11*
Naproxen	9.67 ± 2.00	17.07 ± 2.13*
Nimesulide	20.90 ± 2.64	21.85 ± 3.96
Ibuprofen	39.68 ± 3.96	35.59 ± 3.98
Paracetamol	92.78 ± 7.02	94.58 ± 3.29

* $P<0.05$ compared with phase I.

impairments (Miranda et al., 2007). Then a similar dose–response curve was also obtained and analyzed after the co-administration of dexketoprofen with each NSAID previously identified, in fixed ratio (1:1) combinations based on the mixture of 1/2, 1/4, 1/8, 1/16 of their respective ED_{50} values.

2.3. Isobolographic analysis

Isobolographic analysis was used to characterize drug interactions. The method of isobolographic analysis has been described previously in detail (Miranda et al., 2006). The isobologram was built by connecting the ED_{50} of the dexketoprofen plotted on the abscissa with the ED_{50} of the corresponding NSAID 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:

$$ED_{50}\text{add} = ED_{50}\text{NSAID}/(P1 + R'P2),$$

where R is the potency ratio of the NSAID alone to dexketoprofen alone, $P1$ is the proportion of NSAID and $P2$ is the proportion of dexketoprofen in the total mixture. In the present study, fixed-ratio proportions were selected by first combining the ED_{50} of each compound and then constructing a dose–response curve in which ED_{50} fractions (1/2, 1/4, 1/8 and 1/16) of dexketoprofen with NSAID combinations were administered; in the equation above, ED_{50} add is the total dose and the variance of ED_{50} add was calculated from the fraction of the ED_{50}'s (i.e. 0.5) in the combination as:

$$\text{Var } ED_{50}\text{add} = (0.5)^2\text{Var } ED_{50}\text{NSAID} + (0.5)^2\text{Var } ED_{50} \text{ dexketoprofen}.$$

From these variances confidence limits are calculated and resolved according to the ratio of the individual drugs in the combination. The ED_{50} for the drug combinations was obtained by linear regression analysis of the dose–response curves. A supra-additive or synergistic effect is defined as the effect of a drug combination that is higher and statistically different (ED_{50} significantly lower) than the theoretically calculated equieffect of a drug combination in the same proportion. If the ED_{50}'s are not statistically different, the effect of the combination is additive and additivity means that each constituent contributes with its own potency to the total effect. The interaction rate was calculated as the experimental ED_{50}/the theoretical ED_{50}. If the value is close to 1, the interaction is additive. Values lower than 1 are an indication of the magnitude of supra-additive or synergistic interactions, and values higher than 1 correspond to sub-additive or antagonistic interactions (Miranda et al., 2007).

Table 2
Theoretical and experimental ED_{50} values with 95% confidence limits (CL), ED_{50} (CL), mg/kg i.p., for antinociceptive activity of dexketoprofen combined with other analgesic in phase I and phase II of the orofacial formalin test of mice

Dexketoprofen plus	ED_{50} (CL), mg/kg, Phase I		ED_{50} (CL), mg/kg, Phase II	
	Theoretical	Experimental	Theoretical	Experimental
Naproxen	12.8 (8.9–18.5)	6.1 (4.4–9.4)	33.9 (22.4–47.1)	8.6 (7.0–11.2)
Nimesulide	18.4 (14.2–23.8)	4.2 (3.5–5.0)	36 (26.1–49.7)	13.5 (10.9–16.8)
Ibuprofen	27.8 (21.1–36.7)	6.1 (4.4–9.4)	42.8 (32.2–57.0)	8.6 (7.1–11.2)
Paracetamol	54.4 (41.8–70.7)	1.0 (1.5–0.4)	72.4 (61.5–85.2)	9.9 (6.5–13.4)

All results are significant ($P<0.05$) when compared ED50 (CL) of phase I with phase II.

2.4. Drugs

All drugs were freshly dissolved in a saline solution in a constant volume of 10 ml/kg and were administered intraperitoneally (i.p.).

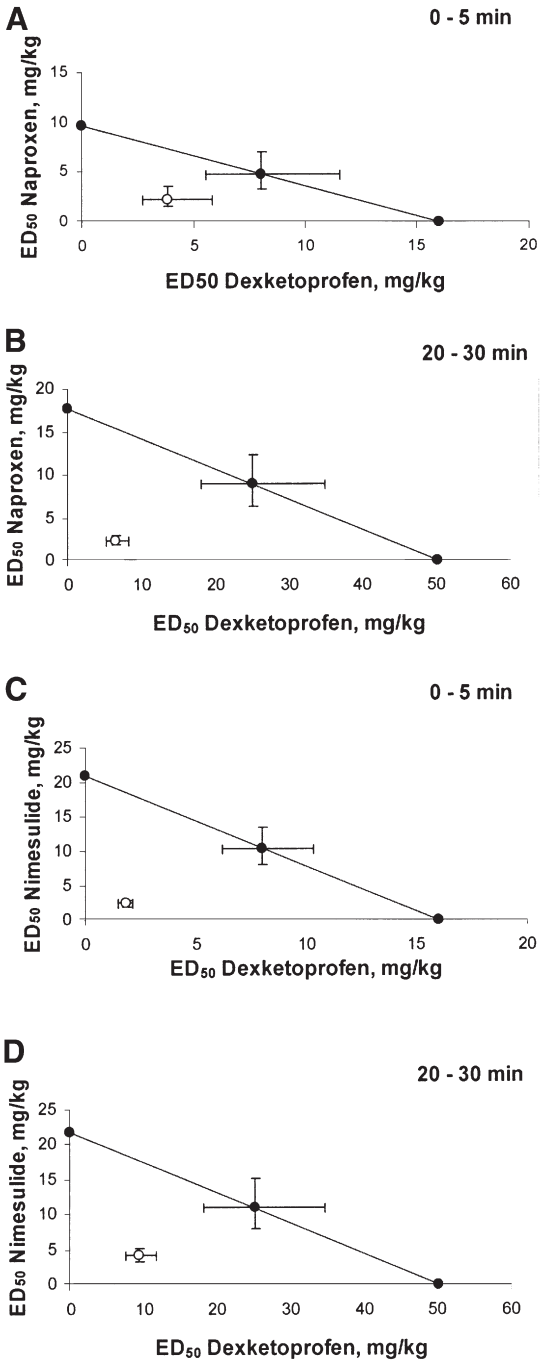

Fig. 3. Isobolograms for the combination of dexketoprofen and naproxen in phase I (1A) and in phase II (1B) and for the combination of dexketoprofen and nimesulide in phase I (1C) and in phase II (1D) of the orofacial formalin test in mice. Filled circles (●) are the theoretical ED_{50}'s with 95% CL and open circles (o), the experimental ED_{50}'s with 95% CL.

Dexketoprofen, ibuprofen, nimesulide or naproxen were administered at doses of 3–300 mg/kg., and paracetamol at doses of 10–300 mg/kg. Dexketoprofen was a gift from Menarini Laboratories, Spain; paracetamol by Bristol–Myers–Squibb, France; nimesulide was purchased from Grunenthal Chilena Ltda; naproxen from Saval Laboratories and ibuprofen from Sigma Chemical Co, USA.

2.5. Statistical analysis

Results are presented as mean±S.E.M. or as ED_{50} values with 95% confidence limits (95% CL). Isobolographic calculations were performed with the program Pharm Tools Pro (version 1.27, the McCary Group Inc.), based on Tallarida (2000). The statistical analysis of the isobolograms was performed according to Tallarida (Tallarida, 2000) and the statistical difference between experimental and theoretical values was assessed by the Student's *t* test for independent means, and the *P* values below 0.05 ($P<0.05$) were considered significant.

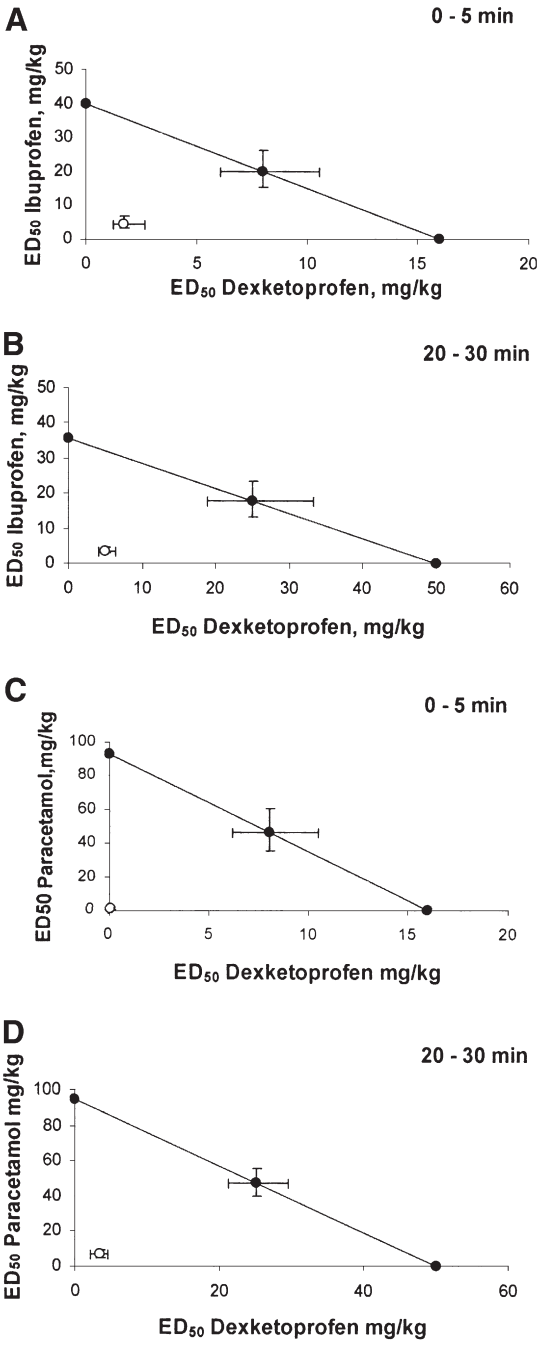

Fig. 4. Isobolograms for the combination of dexketoprofen and ibuprofen in phase I (2A) and in phase II (2B), and for the combination of dexketoprofen and paracetamol in phase I (2C) and in phase II (2 D) of the orofacial formalin test in mice. Filled circles (●) are the theoretical ED_{50}'s with 95% CL and open circles (o), the experimental ED_{50}'s with 95% CL.

Table 3
Interaction indexes and ratio dexketoprofen/analgesic in the phase I and phase II of the orofacial formalin test of mice

Dexketoprofen plus	Interaction index		Ratio Dexketoprofen/analgesic	
	Phase I	Phase II	Phase I	Phase II
Naproxen	0.476	0.254	1:1.65	1:2.83
Nimesulide	0.230	0.374	1:0.76	1:2.29
Ibuprofen	0.220	0.201	1:0.40	1:1.40
Paracetamol	0.019	0.136	1:0.17	1:0.53

The lower value of interaction index indicates higher potency of combination.

3. Results

3.1. Nociceptive behavioral response

The time course of the nociceptive responses to the orofacial formalin test is presented on Fig. 1. This nociceptive response presents a typical biphasic time course with an early and short-lasting, 5 min, first period of activity (Phase I) followed, after a 15 min quiescent period, by a second, prolonged (20–30 min) tonic phase (Phase II).

3.2. Antinociception induced by analgesics

The i.p. administration of dexketoprofen, naproxen, nimesulide, ibuprofen, or paracetamol induced a dose-dependent antinociceptive activity with different potencies either at phase I or in phase II on the orofacial formalin test. The dose response curves of the different NSAIDs are displayed in Fig. 2. The corresponding ED_{50} values are summarized in Table 1.

3.3. Interaction between dexketoprofen and other analgesic drugs

The interactions between the combination of dexketoprofen and naproxen, nimesulide, ibuprofen or paracetamol on the basis of the fixed ratio (1:1) of their ED_{50} values alone were calculated by isobolographic analysis. The theoretical additive ED_{50} values and the experimental ED_{50} values for the fixed ratio combination are shown in Table 2.

Statistical analysis using the data from the isobolographic analysis indicates that synergistic interactions occur between dexketoprofen and each of NSAID examples of different COXs inhibitors. These results are shown in Figs. 3 and 4.

Furthermore, the interaction index values of the combinations demonstrated the following rank of potencies for both phases: dexketoprofen/paracetamol>dexketoprofen/ibuprofen>dexketoprofen/nimesulide>dexketoprofen/naproxen (Table 3).

4. Discussion

The most interesting finding of this study is that the intraperitoneal administration of dexketoprofen, naproxen, nimesulide, ibuprofen, or paracetamol produced a dose-dependent antinociceptive activity in both phases of the orofacial formalin test in mice. The behavioral response of this assay consists of the typical biphasic time course seen in all formalin models. Thus, the phase I results essentially from the direct stimulation of nociceptors, whereas phase II involves a period of sensitization during which inflammatory phenomena occur from peripheral mechanisms (Le Bars et al., 2001). This dose-dependent antinociceptive activity in the orofacial formalin test is

important since for the orofacial pain there is no behavioral nociceptive test currently used in mice and the drugs used are all human analgesics. In addition, these results confirm the antinociceptive activity of the mentioned drugs, in other preclinical assays: i.e., acetic acid writhing test, tail flick test, formalin test (Botting 2003; Luccarini et al., 2006; Matson et al., 2007; Miranda et al., 2006, 2007).

The findings of the present work are important since it has been reported that only higher doses of analgesic agents are required to induce significant antinociception in the first compared to the second phase of the orofacial formalin assay (Raboisson and Dallel, 2004). In this study, this assumption is concordant only with the antinociception induced by dexketoprofen and naproxen. However, according to the ED_{50} values for the antinociceptive activity, nimesulide, ibuprofen and paracetamol have similar potency, both in phase I and phase II, of the orofacial formalin test. It may be noted that paracetamol, a drug that has been considered to be an atypical NSAID, since it is a weak COX inhibitor (Botting, 2003), displays activity in both phases of the orofacial formalin test, with phase II being considered representative of inflammatory pain.

The different combination tested in the orofacial formalin test produced a synergistic interaction. The interaction index, an expression of the magnitude of the interaction, may relate to the COX selectivity in both phases. The results demonstrated that dexketoprofen combined with COX inhibitors induced a synergism with the following rank: COX-3>COX-2>COX-1. These findings are particularly interesting due to the fact that the COX-3 inhibitor, paracetamol, displayed a robust anti-inflammatory activity in the orofacial formalin test. This is an assay of acute and inflammatory pain which mimics some features of inflammatory pain in humans (Luccarini et al., 2004).

As the precise mechanisms of pain control in the orofacial pain are largely unknown, the trigeminal system appears to be engaged (Takemura et al., 2006), the mechanisms responsible for the synergism in the antinociceptive activity of dexketoprofen with NSAIDs are not clear. Numerous possible mechanisms might explain the synergistic interactions among analgesic drugs that involve virtually all levels of cell function (Barrera et al., 2005). In this case, a hypothesis would be for example, dexketoprofen might enhance the affinity of NSAIDs for its respective COX; decrease the rate of elimination of NSAIDs; enhance activation of G-protein with the consequent increase in the activity of NSAIDs, etc. Furthermore, emphasis has been placed on the fact that the use of multiple drugs with different action mechanisms may be the basis of synergism (Chou, 2006).

In conclusion, the data of the present study shows that dexketoprofen combined with NSAIDs produces a synergic antinociceptive activity. These findings may improve the therapeutic profile of this type of combination, especially because with low doses of the components, side effects are not likely to appear, and it is possible to use these combinations especially for long-term pain treatment.

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