INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) have long been the most widely used treatments for pain. All NSAIDs act as inhibitors of the cyclooxygenase (COX) active site of COX isozymes (1, 2).

Morphine (M) is a prototypical opioid and is not only the most effective analgesic known, but also highly addictive drugs of abuse (4). The analgesia induced by M depends on the activation of receptors located peripherally and in the CNS at both spinal and supraspinal level (5).

The potentiation of the antinociception induced by M in several models of pain has been described. Thus, in a model of visceral pain, ketorolac and metamizol induced synergism on the M effect (6, 7) and a similar effect was obtained with M and dipyridone in the formalin test (8). The combination of M and diclofenac has been shown to be synergistic in a model of acute inflammatory pain (9). A synergistic antiallodynic effect of spinal M administered with ketorolac in nerve injured rats has also been reported (10). Recently, it has been shown that the antinociceptive activity of M combined with different NSAIDs, induced a supra-additive interaction (synergy) in a model of mice visceral acute pain (11).

However, the involvement of opioid receptors (MOR, KOR, DOR, or NOR) in these interactions have been not extensively studied, with the exception of the work of Miranda et al. (11), where the coadministration of M and NSAIDs was done by intraperitoneal route.

The clinical importance of the combination of NSAIDs with M is evident, since they are considered in the step 3 of the WHO ladder for pain treatment. In addition, if the simultaneous administration of M and NSAIDs is recommended, the potential advantages of a synergistic lies in the potentiated increased analgesic effect with reduction of drug-induced adverse reactions.

MATERIAL AND METHODS

Animals

Male CF-1 mice (30 g), housed on a 12 h light - dark cycle at 22 ± 2 °C and with access to food and water ad libitum were
used. Experiments were performed in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigation of experimental pain approved by the Animal Care and Use Committee of the Faculty of Medicine, University of Chile. Animals were acclimatized to the laboratory for at least 2 h before testing, were used only once during the protocol and were sacrificed immediately after the algesiometric test. The number of animals was kept at a minimum compatible with consistent effects of the drug treatments.

**Experimental protocols**

Experiments were carried out in three groups. First group was performed to determine the effect of control animals (6% glucose) and were run interspersed concurrently with drug treatment. Second group was executed to evaluate the antinociceptive activity of M and NSAIDs and in the third group performed to determine the effect naltrindole on the interactions of M with each NSAIDs.

Preliminary experiments determined that the same volume of vehicle of M, NSAIDs and naltrindole did not alter antinociceptive responses.

**Intrathecal injections**

For intrathecal (i.t.) injections, the animals were restrained manually and a 50 µL Hamilton syringe with a 26-gauge needle was inserted into the subdural space between L5 and L6, according to a previously described technique (16). The doses were administered in a constant volume of 5 µL, dissolved in a slightly hyperosmotic solution of glucose (6%) to limit rapid diffusion of the drug to higher levels of the spinal cord. The withdrawal of the tail during the insertion of the needle is indicative of a successful spinal administration. Control animals (6% glucose) were run interspersed concurrently with the drug treatments.

**Measurement of analgesic activity**

Analgesic activity was assessed by the writhing test, a chemical visceral pain model (16, 17). Mice were injected intraperitoneally (i.p.) with 10 mL/kg of 0.6% acetic acid solution, 15 min after the intrathecal (i.t.) administration of the drugs, a time at which preliminary experiments showed occurrence of the maximum effect. A writh is characterized by a wave of contraction of the abdominal musculature followed by the extension of the hind limbs. The number of writhes in a 5 min period was counted, starting 5 min after acetic acid administration. Antinociceptive activity was expressed as percent inhibition of the number of writhes observed in control animals (19.6 ± 0.30, n = 60).

**Protocol**

Dose-response curves for morphine (M), diclofenac (DICLO), ketoprofen (KETO), meloxicam (MELO), metamizol (META), naproxen (NAPRO), nimesulide (NIME), parecoxib (PARE) and piroxicam (PIRO) were obtained using at least six animals at each of at least four doses. A least-squares linear regression analysis of the log dose-response curve allowed the calculation of the dose that produced 50% of antinociception (ED50) for each drug alone. Then a dose-response curve was also obtained by the coadministration of M with each NSAID, given as a mixture, in combinations of fixed ratios based on fractions of their respective ED50 values: 1/2, 1/4, 1/8, 1/16. Isobolographic analysis was used to identify the participation of DOP opioid receptors in the antinociceptive interaction of M and NSAIDs, mice were pretreated i.t. with NTI (30 µg/mice) 30 min before the administration of the combinations.

The method for isobolographic analysis has been described previously in detail (17). Supra-additivity or synergistic effect is defined as the effect of a drug combination that is higher and statistically different (ED50 significantly lower) than the theoretical calculated equieffect of a drug combination with the same proportions. If the ED50’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 (18). The interaction index was calculated as experimental ED50 / theoretical ED50. 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 (18).

**Drugs**

The following NSAID were freshly dissolved in a slightly hyperosmotic solution of glucose (6%) to limit diffusion and were provided by local pharmaceutical companies: diclofenac by Novartis Chile S.A., ketoprofen by Rhone-Poulenc Rorer, meloxicam by Laboratorios Saval S.A., metamizol by Sanderson S.A., naproxen by Laboratorios Saval S.A., nimesulide by Grúhenthal Chile Ltda., parecoxib and piroxicam by Pfizer Chile. Morphine hydrochloride and naltrindole hydrochloride were purchased from Sigma Chemical Co (St. Louis, MO, USA).

**Statistical analysis**

Results are presented as mean S E M and as ED50 values with 95% confidence limits (95 % CL). The statistical difference between theoretical and experimental values of isobolograms was assessed by Student’s t-test for independent means. The program used to perform procedures was Pharm Tools Pro (version 1.27, The McCary Group Inc.). P values less than 0.05 (P < 0.05) were considered significant.

**RESULTS**

**Antinociception induced by M and NSAIDs**

The i.t. administration of M (0.01,0.1,0.3 and 1 µg/mice) and the following NSAIDs: MELO and NIME (1,3,10 and 30 µg/mice); DICLO, NAPRO, PIRO, PARE, META and KETO (3, 10, 30 and 100 µg/mice) produced dose-dependent

<table>
<thead>
<tr>
<th>Drugs</th>
<th>ED50 µg/mice i.t. (95% CL)</th>
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<tbody>
<tr>
<td>Morphine</td>
<td>0.0054 (0.0027 - 0.102)</td>
</tr>
<tr>
<td>Meloxicam</td>
<td>6.3 (5.7 - 7.5)</td>
</tr>
<tr>
<td>Nimesulide</td>
<td>8.7 (6.3 - 11.7)</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>12.9 (12.0 - 13.8)</td>
</tr>
<tr>
<td>Naproxen</td>
<td>14.4 (11.1 - 18.6)</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>15.3 (12.6 - 18.6)</td>
</tr>
<tr>
<td>Parecoxib</td>
<td>18.6 (12.9 - 28.2)</td>
</tr>
<tr>
<td>Metamizol</td>
<td>24.0 (12.0 - 46.5)</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>25.8 (18.3 - 33.3)</td>
</tr>
</tbody>
</table>

Values are ranked in descending order of potency.
antinociceptive activity with equal efficacy but with different potencies in the writhing test of mice. The ED$_{50}$ values and 95% confidence limits (CL) for the antinociceptive effects of M and NSAIDs are shown in Table 1.

Isobolographic analysis of M / NSAIDs interactions

The antinociceptive activity of the combinations of M with each NSAIDs at fixed ratios of ED$_{50}$ fractions was assessed by an isobolographic analysis of the dose-response curves obtained after i.t. administration. The isobolograms indicate that a

synergistic interactions occurred between M and NSAIDs, as can be see in Fig. 1 and Fig. 2. Table 2 shows the experimental and the theoretical additive ED$_{50}$ values for the combinations with their 95% CL and the combinations fixed ratios. The ratios of the combinations were: NIME/M (1:161); MELO/M (1:1216); DICLO/M (1:2400); NAPRO/M (1:2683); PIRO/M (1:2833); PARE/M (1:3477); META/M (1:4444); and KETO/M (1:4778).

In addition, the interaction index values of the combinations demonstrated the following rank of potencies for the synergic combinations: M/PARE > M/NAPRO > M/META > M/DICLO > M/PIRO > M/MELO < M/NIME < M/KETO (Table 3).
of the combinations of 5.4 µg/mice of morphine (M) with NSAIDs, administered i.t. in the acetic acid writhing test.

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>ED$_{50}$ µg/mice (95%CL)</th>
<th>Experimental</th>
<th>After NTI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meloxicam/M</td>
<td>3.27 (2.70-3.90)</td>
<td>1.72 (1.20-2.10)</td>
<td>1.62 (0.90-1.70)</td>
</tr>
<tr>
<td>Nimesulide/M</td>
<td>4.35 (3.30-5.40)</td>
<td>2.22 (1.50-3.30)</td>
<td>2.40 (1.80-3.90)</td>
</tr>
<tr>
<td>Diclofenac/M</td>
<td>6.48 (4.50-9.90)</td>
<td>2.85 (1.80-6.00)</td>
<td>2.31 (1.80-3.00)</td>
</tr>
<tr>
<td>Naproxen/M</td>
<td>7.23 (6.00-8.70)</td>
<td>2.55 (1.80-3.30)</td>
<td>3.00 (2.10-4.80)</td>
</tr>
<tr>
<td>Piroxicam/M</td>
<td>7.65 (6.00-9.30)</td>
<td>3.51 (2.70-4.50)</td>
<td>3.69 (2.70-5.10)</td>
</tr>
<tr>
<td>Parecoxib/M</td>
<td>9.36 (6.72-12.00)</td>
<td>3.24 (2.40-4.20)</td>
<td>4.65 (3.30-6.90)</td>
</tr>
<tr>
<td>Metamizol/M</td>
<td>12.00 (7.50-18.90)</td>
<td>4.98 (3.00-10.2)</td>
<td>3.75 (3.00-4.50)</td>
</tr>
<tr>
<td>Ketoprofen/M</td>
<td>12.90 (9.00-18.30)</td>
<td>7.32 (6.00-9.60)</td>
<td>4.74 (4.20-5.40)</td>
</tr>
</tbody>
</table>

All experimental values are significant (p< 0.05) compared with theoretical additive values, but not significant compared to NTI values.

<table>
<thead>
<tr>
<th>Interaction Index (I.I.)</th>
<th>Drug combination</th>
<th>Experimental</th>
<th>After NTI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARE</td>
<td>0.345</td>
<td>0.497</td>
<td></td>
</tr>
<tr>
<td>NAPRO</td>
<td>0.353</td>
<td>0.415</td>
<td></td>
</tr>
<tr>
<td>METAMIZOL</td>
<td>0.417</td>
<td>0.313</td>
<td></td>
</tr>
<tr>
<td>DICLOFENAC</td>
<td>0.440</td>
<td>0.357</td>
<td></td>
</tr>
<tr>
<td>PAREOXIB</td>
<td>0.459</td>
<td>0.484</td>
<td></td>
</tr>
<tr>
<td>MELOXICAM</td>
<td>0.500</td>
<td>0.387</td>
<td></td>
</tr>
<tr>
<td>NAPROXILIDE</td>
<td>0.512</td>
<td>0.549</td>
<td></td>
</tr>
<tr>
<td>KETORPROFEN</td>
<td>0.570</td>
<td>0.367</td>
<td></td>
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</tbody>
</table>

Interaction Index (I.I.) listed in ascending order. Lower values indicate higher potency of the drug combinations.

**Effect of naltrexone on the M/NSAIDs interactions**

Neither i.t. administration of NTI (30 µg/mice) nor pretreatment with δ-opioid receptor antagonist had any effect on the antinociceptive activity of i.t. NSAIDs, or in the analgesic effect of i.t. M in the writhing test assay (data not shown). However, the pretreatment of mice with 30 µg/mice of NTI was not able to modify significantly the magnitude of the interaction of the combinations of M and NSAIDs (Fig.1, 2, Table 3).

**DISCUSSION**

The i.t. administration of M and the following NSAIDs, DICLO, KETO, MELO, META, NAPRO, NIME, PARE and PIRO produced a dose-dependent antinociceptive effect in the chemical visceral-somatic assay of the acetic acid writhing test (11, 17). The synergy observed with the isobolographic analysis of the combinations was not significantly modified by NTI. The differences in the magnitude of the interactions observed in the present study as measured by the values of interaction index, may be indirectly related with the COXs selectivity reported by Warner and Mitchell (2). Also, a similar relationship may be found with the ED$_{50}$'s values of their relative potencies. Since all NSAIDs are highly lipophilic drugs (19), differences in their kinetics properties (20, 21) should not contribute to the magnitude of the interaction with M.

PARE, the pro-drug of the selective COX-2 inhibitor valdecoxib (22), is effective when given i.t. and this finding agrees with our previous work (23) indicating that this prodrug can be hydrolyzed to a certain extent in the spinal cord. Cytochrome oxidase activity is present in neurons and glial cells of the spinal cord in various mammalian species (24, 25). In addition, PARE administered by intra-articular injections has shown anti-inflammatory activity in osteoarthritic rats (26), suggesting that the liver may not be the only site of parecoxib enzymatic hydrolysis to valdecoxib.

The findings of the present study related to the action of NTI are concordant with previous reports in which NTI did not reverse the analgesic effects of intrathecal M (27) or the opioid receptor agonists endomorphin-1 (28). Also, it has been reported that the antinociceptive effect of the opioid ligand, oxymorphazol, administered intrathecally was blocked by NTI in the tail-flick assay, but not in the writhing test (29). A similar lack of effect of naltrexone, a MOR-opioid receptor antagonist, on the antinociceptive synergism between intrathecal M and NSAIDs has been previously reported (16). These previous reports and the present results suggest that antinociception following intrathecal morphine involves spinal and supraspinal opioid receptors.

The failure of NTI to influence the synergism of the M/NSAIDs combinations is concordant with the lack of effect of this DOR-opioid antagonist obtained when the drugs were administered i.p (11) and reflects a scanty participation of δ-opioid receptors in the antinociception induced by the coadministration of M with NSAIDs in this model of experimental acute pain.

Although the antinociceptive efficacy of opioids and NSAIDs has been established in several preclinical and clinical studies, their precise mechanism of action have not been determined. Opioids act through the activation of receptors located in the periphery and in the CNS at both spinal and supraspinal levels (4, 5). NSAIDs produce analgesia mainly by inhibition of prostaglandins biosynthesis (1, 2) even when additional mechanisms participate (30-33). The involvement of these additional mechanisms, specially the activation of the NO-cGMP pathway, seems to have more importance than the activation of DOR receptors in the generation of the synergy. Furthermore, recently changes in dopamine metabolism it has been reported in the synergism of morphine-induced analgesia by the neuroprotective substance, 1-methyl-1,2,3,4-tetrahydrosquinoine, an endogenous compound present in the central nervous system of mammals and humans (34).

On the other hand, molecular mechanisms underlying the nature of spinal synergism in acute inflammatory pain, as the used in this work, is still poorly understood. However, it has been reported the importance of immune response-and microglia activation-related genes and that the increased expression of calcitonin gene related peptide (CGRP) could be associated with neuropathic pain (35). In agreement with this assumption, it may...
be hypothetically assumed that decreased expression of CGRP could be linked with the acute inflammatory pain.

In conclusion, the data of the present study demonstrated that M combined with NSAIDs, produce a supra-additive or synergic analgesic effect, that is not related with the activation of DOR opioid receptor subtype. Therefore, these combinations are a viable alternative to clinical pain management, especially trough multimodal analgesia.

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Conflict of interests: None declared.

REFERENCES

5. Dionne RA, Lepinski AM, Gordon SM, Jaber L, Brahim JS, Lopez and A. Correa is gratefully acknowledged.