

The Combination of Naproxen and Citral Reduces Nociception and Gastric Damage in Rats

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It has been shown that the association of non-steroidal anti-inflammatory drugs with plant extracts can increase their antinociceptive activity, allowing the use of lower doses and, thus, limiting side effects. Therefore, the aim of this study was to examine the effects of the interaction between naproxen and citral on nociception and gastric injury in rats. Naproxen, citral, or combinations of naproxen and citral produced an antinociceptive effect. The administration of naproxen produced significant gastric damage, but this effect was not obtained with either citral or the naproxen-citral combination. The ED₅₀ value was estimated for the individual drugs and an isobologram was constructed. The derived theoretical ED₅₀ for the antinociceptive effect (423.8 mg/kg) was not significantly different from the observed experimental value (359.0 mg/kg); hence, the interaction between naproxen and citral mediating the antinociceptive effect is additive. These data suggest that the naproxen-citral combination interacts at the systemic level, produces minor gastric damage, and potentially has therapeutic advantages for the clinical treatment of inflammatory pain.

Key words: Naproxen, Citral, Synergism, Nociception, Rats, Gastric damage

INTRODUCTION

Pain relief can be achieved by a variety of methods, with drug use being the cornerstone of analgesic treatment. The clinical use of combinations of analgesic agents has increased significantly in the last few decades. The purpose of this study was to combine two drugs with different mechanisms of action in order to achieve a synergistic interaction, yielding a sufficient antinociceptive effect with lower doses, and, therefore, reduce the intensity and incidence of untoward effects (Curatolo and Svetcic, 2002). Currently, many different classes of drugs serve as an effective complement to non-steroidal anti-inflammatory drugs (NSAIDs) or opioids in the management of pain. Opioids have

frequently been used in combination with NSAIDs for the clinical management of both acute and chronic pain (Cooper et al., 1982; Minotti et al., 1989; Sun et al., 1993; Collins et al., 2000; Smith et al., 2001; Schnitzer, 2003; Oldfield and Perry, 2005); this limits the doses of medication that a patient can receive.

There are several studies that have evaluated the possible interactions between herbs and drugs. Lala et al. (2004) evaluated the pharmacokinetic and pharmacodynamic interactions between diclofenac and Trikatu (an Ayurvedic formulation composed of the dried fruits of *Piper nigrum* and *Piper longum* and the dried rhizomes of *Zingiber*). The authors found that the extent to which edema was inhibited by the combination of diclofenac and Trikatu was similar to that shown by Trikatu alone, but significantly less than that produced by the sole administration of diclofenac. Likewise, Trikatu significantly decreased the plasmatic concentrations of diclofenac. Recently, a synergistic interaction between *Heliopsis longipes* (an herbaceous plant found in Mexico) and diclofenac was demonstrated in

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the Hargreaves model of thermal hyperalgesia in the mouse (Acosta-Madrid et al., 2009). More recently, our group found a synergism between a combination of the NSAID, naproxen, and citral in the anti-inflammatory effects in rats (Ortiz et al., 2010). Citral (3,7-dimethyl-2,6-octadienal) is a monoterpene that occurs naturally in herbs, plants, and citrus fruits. It is a natural mixture of the isomeric acyclic aldehydes, geranial (trans-citral, citral A) and neral (cis-citral, citral B). Recently, it was demonstrated that citral produces a long-lasting inhibition of TRPV1–3 and TRPM8 and a transient block of both TRPV4 and TRPA1 (Stotz et al., 2008). Similarly, it was demonstrated that the main constituent of the essential oil of *Cinnamomum insularimontanum* is citral, and that this compound exerts a significant inhibitory effect on the production of nitric oxide in lipopolysaccharide-stimulated RAW 264.7 cells (Lin et al., 2008). Moreover, citral exhibited an anti-inflammatory effect in a test of croton oil-induced ear edema in mice (Lin et al., 2008). Therefore, in light of the anti-inflammatory effects and inhibition of ion channels, we decided to evaluate the antinociceptive effect and assess the gastric damage resulting from the systemic administration of citral, naproxen, and combined citral-naproxen in rats.

MATERIALS AND METHODS

Animals

Male Wistar rats aged 7–9 weeks (weight range: 180–220 g) from our own breeding facilities were used in this study. Efforts were made to minimize animal suffering and to reduce the number of animals used. Each rat was used in only one experiment and sacrificed in a CO₂ chamber at the end of the experiment. All experiments followed the Guidelines on Ethical Standards for Investigation in Animals (Zimmermann, 1983), and the protocol was approved by the Institutional Animal Care and Use Committee (CINVESTAV, IPN).

Drugs

Citral, naproxen, and formaldehyde were purchased from Sigma. Citral was dissolved in a 10% Tween-20 solution. Naproxen was dissolved in saline.

Measurement of antinociceptive activity

Pain and antinociception were assessed using the previously described formalin test (Jiménez-Andrade et al., 2003; Ortiz et al., 2003; Ortiz and Castañeda-Hernández, 2008). Briefly, fifty microliters of diluted formalin (1%) were injected subcutaneously (s.c.) into

the plantar surface of the right hind paw, and the resulting flinching behavior was considered to be an expression of nociception. Graphs of the numbers of flinches against time were constructed, and the resulting curves were biphasic. After the initial acute phase (0–10 min), there was a short quiescent period, followed by a prolonged tonic response (15–60 min). The area under the curve for both phases was estimated, and a significant reduction in the area was interpreted as an antinociceptive effect.

Sixty min before the formalin insult, animals were orally injected with vehicles or increasing doses of naproxen (10–300 mg/kg), citral (30–1000 mg/kg), or the naproxen-citral combination (53.0, 106.0, 211.9, and 423.8 mg/kg). The person performing these experiments was unaware of the treatments that the rats had received. The injection volumes were 4 mL/kg. Rats in all groups were observed for changes in behavioral or motor function that could have been induced by the treatments. The ability of the animals to stand and walk with a normal posture was assessed, but this was not quantified.

Gastric damage

Following completion of the formalin experiments (3 h after), each rat was euthanized in a CO₂ chamber. The stomach was removed and the extent of hemorrhagic damage was scored by an observer who was unaware of the treatments that each rat had received. The length (in mm) of all hemorrhagic lesions was measured and the gastric damage score for each rat stomach was calculated by summing these values (Wallace et al., 2000).

Data analysis

Results are presented as mean \pm S.E.M. for 6–8 animals per group. The time courses of the antinociceptive responses resulting from the administration of the individual drugs and the drug combination were constructed by plotting the mean number of flinches as a function of time. The areas under the resulting curves (AUC) were calculated using the trapezoidal rule. AUC was calculated for the two phases of the assay and the percent of antinociception for each phase was calculated according to the following equation (Ortiz and Castañeda-Hernández, 2008): $Percent\ of\ antinociception = [(AUC_{vehicle} - AUC_{post\ compound}) / AUC_{vehicle}] \times 100$.

Dose-response curves were constructed using least-squares linear regression, and the antinociceptive ED₅₀ \pm S.E. value was calculated according to Tallarida (2000). The interaction between citral and naproxen was characterized by isobolographic analysis in which

it was assumed that the combinations are comprised of equieffective doses of the individual component drugs. Thus, from the dose-response curves of each individual agent, the dose resulting in 50% of the effect (ED_{50}) could be determined. Subsequently, a dose-response curve was obtained by concurrent delivery of two drugs (citral plus naproxen) in a fixed-ratio mixture (1:1) that was based on the ED_{50} values of each individual agent. To construct the experimental antinociceptive effect-dose curve, each group of rats received one of the following doses of the combination: naproxen $ED_{50}/2$ (136.4 mg/kg) + citral $ED_{50}/2$ (287.4 mg/kg); naproxen $ED_{50}/4$ (68.2 mg/kg) + citral $ED_{50}/4$ (143.7 mg/kg); naproxen $ED_{50}/8$ (34.1 mg/kg) + citral $ED_{50}/8$ (71.85 mg/kg); or naproxen $ED_{50}/16$ (17.05 mg/kg) + citral $ED_{50}/16$ (35.9 mg/kg).

The experimental ED_{50} value for the naproxen-citral combination was calculated from this curve.

The theoretically additive effect of the antinociceptive ED_{50} was estimated from the dose-response curves obtained by sole administration of each drug (i.e., considering that the effect observed with the combination is the result of the sum of the effects of each individual drug). This theoretical ED_{50} value was then compared with the experimentally derived ED_{50} value to determine whether there was a statistically significant difference (Tallarida et al., 1999; Tallarida, 2002). The theoretical and experimental ED_{50} values of the combinations were also contrasted by calculating the interaction index (g) as follows: $g = ED_{50} \text{ of combination (experimental)} / ED_{50} \text{ of combination (theoretical)}$. An interaction index that was not significantly different from unity corresponds to an additive interaction, whereas values higher or lower than unity imply an antagonistic or synergistic interaction, respectively (Tallarida, 2002; Jiménez-Andrade et al., 2003; Ortiz and Castañeda-Hernández, 2008).

Statistical analysis

Dose-response data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's test for post hoc comparison. The statistical significance of differences between the theoretical additive ED_{50} value and the experimentally derived ED_{50} value were determined using Student's t test (Tallarida, 2000). An experimental ED_{50} value that was significantly lower than the theoretical additive ED_{50} value was regarded as an indication of a synergistic interaction between citral and naproxen. Statistical significance was considered to be achieved when $p < 0.05$.

RESULTS

Systemic antinociceptive effect of naproxen and citral

The administration of formalin produced a typical pattern of flinching behavior. The initial phase started immediately after the administration and then diminished gradually over the next 10 min. The second phase started after 15 min and lasted until 1 h-post administration. Naproxen and citral produced a dose-dependent antinociceptive effect during phase two ($p < 0.05$; Fig. 1) but not during phase one ($p > 0.05$; data not shown). None of the treatments produced a significant alteration of ambulation or motor activity.

Antinociceptive interaction of naproxen and citral after systemic administration

The ED_{50} values for systemic naproxen and citral in phase 2 of the formalin test were 272.8 ± 38 mg/kg and 574.8 ± 121.4 mg/kg, respectively. Fixed-dose ratio combinations were prepared as described in the

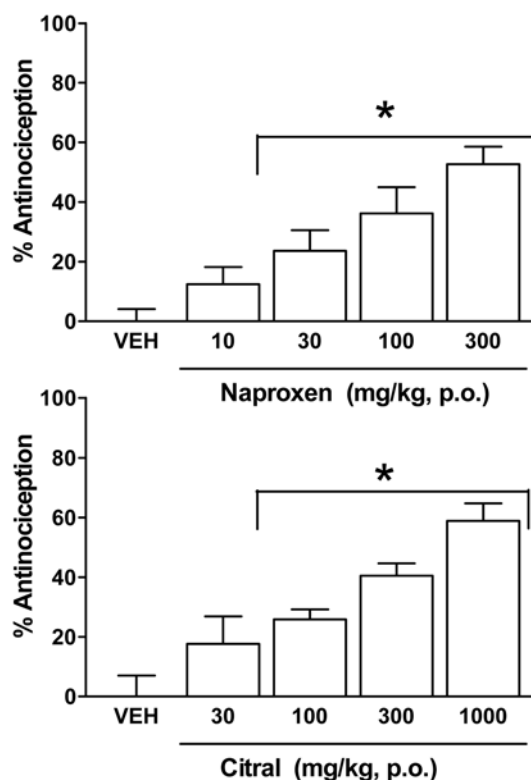


Fig. 1. Systemic antinociceptive effect of naproxen and citral on the 1% formalin test. Prior to the injection of formalin, rats were systemically pretreated with the vehicle (VEH), naproxen, or citral. Data are expressed as the percent of antinociception on the second phase. Each point corresponds to the mean \pm S.E.M. of 6-8 animals. *Significant difference from vehicle group ($p < 0.05$) as determined by the analysis of variance followed by Dunnett's test.

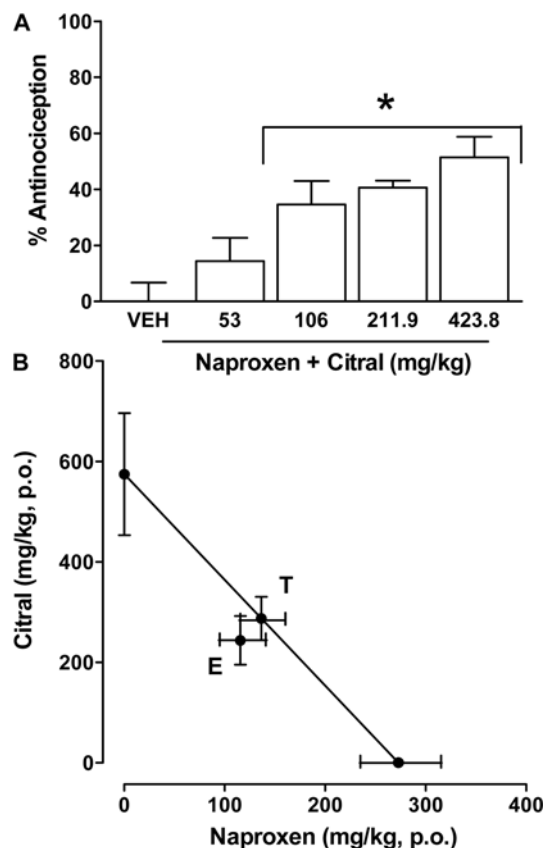


Fig. 2. Systemic antinociceptive effect of the naproxen-citral combination. (A) Rats were systemically pretreated, before the formalin infection, with the vehicle (VEH) or the naproxen-citral combination. (B) Isobologram showing the systemic interaction between naproxen and citral on the formalin test. The oblique line, between the x and y axes, is the theoretical additive line. The point in the middle of this line, denoted by "T", is the theoretical additive point calculated from the individual drug ED_{50} values. The experimental point, denoted by "E", is the actual observed ED_{50} value for this combination. Horizontal and vertical bars indicate the S.E.M.

methods section, and these were assayed in order to construct a dose-response curve for the naproxen-citral combination. The corresponding experimental ED_{50} was 359.0 ± 71.7 mg/kg (Fig. 2). This value was not significantly lower ($p > 0.05$) than the theoretical ED_{50} , which was predicted to be 423.8 ± 63.6 mg/kg; this similarity is clearly shown in Fig. 2, as the experimental ED_{50} is located near to the additive dose line. Furthermore, the interaction index (g) for the naproxen-citral combination (0.85 ± 0.2) was not statistically different from unity. Thus, these data suggest that, at the systemic level, the interaction between the antinociceptive actions of naproxen and citral is additive.

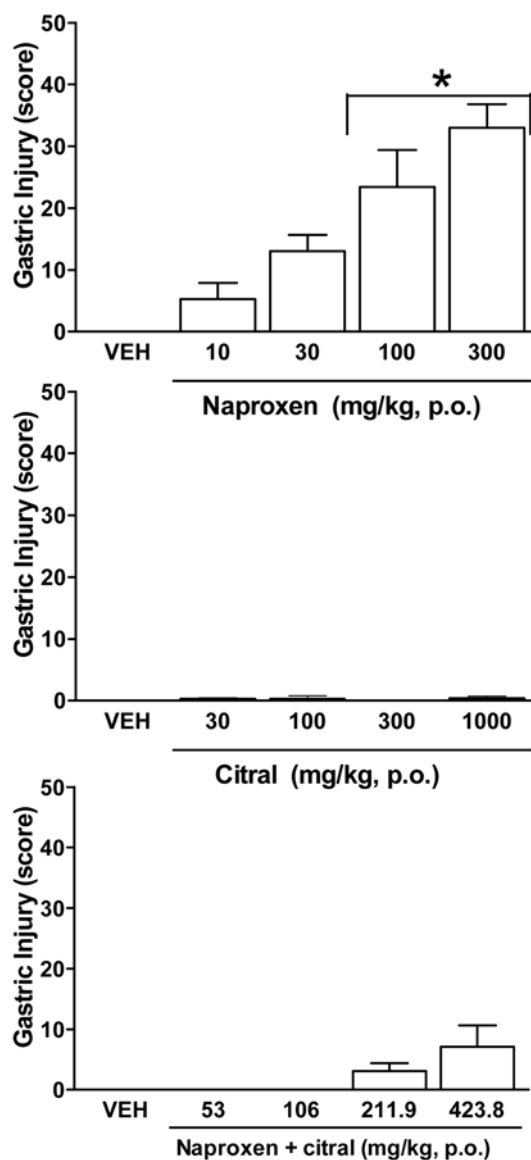


Fig. 3. Gastric injury produced by naproxen, citral, and the naproxen-citral combination. Rats were pretreated with an oral administration of vehicle (VEH), naproxen, citral, or a naproxen-citral combination; they were sacrificed three hours later. The stomach was removed and the extent of hemorrhagic damage was scored. Data are expressed as the score of gastric injury. Each point corresponds to the mean \pm S.E.M. of 6-8 animals. *Significantly different from the vehicle group ($p < 0.05$) as determined by the analysis of variance followed by Dunnett's test.

Gastric damage

At hour three, oral administration of naproxen, but not citral, resulted in the formation of hemorrhagic erosions in the corpus of the stomach (Fig. 3). Gastric damage caused by combined citral and naproxen administration was not different from that measured in the vehicle-treated animals ($p > 0.05$) (Fig. 3).

DISCUSSION

Antinociception of naproxen, citral and the naproxen-citral combination

In the formalin test, diluted formaldehyde was injected subcutaneously into a hind paw and nociceptive behavior was scored. Two phases of response are typically observed (Dubuisson and Dennis, 1977). Opioid analgesics, such as morphine, seem to exert an antinociceptive effect in both phases (Karim et al., 1993). In contrast, NSAIDs such as naproxen seem to suppress only the second phase (Malmberg and Yaksh, 1992). Naproxen is a very potent anti-inflammatory drug that is used for treating painful conditions, such as arthritis and gout (Brogden et al., 1979). However, like other NSAIDs, its use is limited by a relatively high incidence of adverse effects; the most common of these are gastrointestinal ulceration and bleeding (Bjarnason and Thjodleifsson, 1999). In the present study, the systemic administration of naproxen was able to decrease the nociceptive effect induced by formalin. The anti-inflammatory and antinociceptive properties of naproxen have been attributed to the inhibition of cyclooxygenase, in both the central nervous system and peripheral tissues, and the consequent inhibition of prostaglandin biosynthesis (Tavares et al., 1985). Therefore, it is probable that inhibition of prostaglandin synthesis is responsible for the antinociceptive effect observed with the formalin.

Recently, McNamara et al. (2007) demonstrated that formalin excites sensory neurons by directly activating TRPA1, a cation channel that plays an important role in inflammatory pain. Moreover, they showed that the blockade of TRPA1 channels *in vivo* (using either a specific antagonist or via disruption of the TRPA1 gene) substantially attenuated the pain-related response to formalin. In the present study, the formalin test demonstrated that the systemic administration of citral caused a dose-dependent antinociceptive action in the rat. It was previously shown that citral inhibits TRPV1–4, TRPM8, and TRPA1 channels (Stotz et al., 2008). Therefore, it is highly likely that the antinociceptive action induced by citral could be due to the inhibition of TRPA1 channels, but it is also possible that other mechanisms might be involved. For example, it has been demonstrated that citral is able to inhibit the production of nitric oxide in lipopolysaccharide-stimulated RAW 264.7 cells (Lin et al., 2008), suppress COX-2 expression (Katsukawa et al., 2010), and decrease lymphocyte migration by inhibiting beta7-integrin expression (Watanabe et al., 2010). It remains to be determined which of these mechanisms are actually involved in the antinociceptive effect.

To the best of our knowledge, this is the first study to report an antinociceptive effect of citral.

The isobolographic analysis presented in this study demonstrated an additive interaction between naproxen and citral in systemic antinociception. In previous studies, the delivery of naproxen and other analgesics were shown to be markedly synergistic (Hurley et al., 2002; Satyanarayana et al., 2004; Miranda and Pinardi, 2004; Miranda et al., 2005, 2006). For example, the oral administration of naproxen and gabapentin in a fixed-dose ratio synergistically suppressed thermal hyperalgesia in the carrageenan model of peripheral inflammation (Hurley et al., 2002). Likewise, intraperitoneally or intrathecally administered analgesics (paracetamol and morphine, respectively), in combination with naproxen, synergistically interact to reduce pain in the acetic acid abdominal constriction test in mice (writhing test) (Miranda et al., 2005, 2006). However, our study demonstrates that not all naproxen and analgesic interactions are synergistic (Hurley et al., 2002; Tallarida et al., 2003; Miranda and Pinardi, 2004). Furthermore, an additive response was also obtained with the concurrent intrathecal delivery of naproxen and clonidine in the writhing test (Miranda and Pinardi, 2004), and the combination of glucosamine and naproxen also resulted in an additive antinociceptive interaction in the same test (Tallarida et al., 2003). It might be that this discrepancy resulted because both the hyperalgesic and nociceptive effects and the edematous constituents vary according to the models (species, tissues, type and intensity of the injurious stimuli) used.

Gastric injury induced by naproxen, citral and the naproxen-citral combination

NSAIDs are among the most widely used medications in the world. However, the vast majority of NSAIDs cause substantial gastrointestinal injury. For this reason, several strategies have been adopted to reduce the risk of NSAID-induced upper gastrointestinal complications, including reducing the NSAID dose, switching to NSAIDs that are perceived to be less toxic, and the concomitant use of gastroprotective agents (Rahme et al., 2004). On furthermore, patients with arthritis or other conditions that necessitate chronic pain relief should receive the NSAID that is safest from a cardiovascular perspective (for example, naproxen; Kearney et al., 2006). In this study, one of the main objectives was to evaluate the gastric injury produced by naproxen, citral, and the naproxen-citral combination, but we did not evaluate the gastroprotective effect of citral on the gastric damage induced by naproxen. Therefore, consistent with earlier evidence (Bjarnason and

Thjodleifsson, 1999), we found that naproxen was able to produce significant gastric injury by the 3 h time-point. However, a surprising observation was that citral alone produced insignificant gastric damage in all animals by the 3 h time-point, and the naproxen-citral combination produced less gastric injury than naproxen alone. The gastric damage score, determined after administration of the highest doses of combined naproxen and citral (136.4 mg/kg and 287.4 mg/kg, respectively) at 3 h was 7.1 ± 2.9 , which is less than the gastric injury resulting from sole administration of 100 mg/kg of naproxen at 3 h (23.4 ± 6.0). These results have provided important insight into the safety of citral. We propose that systemic citral does not promote gastric damage, and it does not interfere with gastroprotective factors.

Several studies have demonstrated the anti-inflammatory and analgesic activities of herbal extracts and their constituents (Chrubasik and Pollak, 2002; Chrubasik et al., 2007). In addition, there is evidence that phytogetic agents have traditionally been used by herbalists and indigenous healers for the prevention and treatment of peptic ulcers (Borrelli and Izzo, 2000). Therefore, the interaction observed in this study suggests that the combined use of naproxen and citral has fewer gastrointestinal and renal side-effects profile than naproxen alone. Further research into the clinical efficacy, safety, and benefits of this combination in humans is needed.

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