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Research Article

Supra-Additive Interaction of Docosahexaenoic Acid and Naproxen and Gastric Safety on the Formalin Test in Rats

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ABSTRACT The aim of this work was to evaluate the effect of docosahexaenoic acid (DHA) on the pharmacokinetics and pharmacodynamics—nociception—of naproxen in rats, as well as to determine the gastric safety resulting from this combination versus naproxen alone. Female Wistar rats were orally administered DHA, naproxen or the DHA-naproxen mixture at fixed-ratio combination of 1:3. The antinociceptive effect was evaluated using the formalin test. The gastric injury was determined 3 h after naproxen administration. An isobolographic analysis was performed to characterize the antinociceptive interaction between DHA and naproxen. To determine the possibility of pharmacokinetic interactions, the oral bioavailability of naproxen was evaluated in presence and absence of oral DHA. The experimental effective dose ED_{30} values (*Zexp*) were decreased from theoretical additive dose values (*Zadd*; $P < 0.05$). The isobolographic analysis showed that the combination exhibited supra-additive interaction. The oral administration of DHA increased the pharmacokinetic parameter $AUC_{0\text{-}t}$ of naproxen ($P < 0.05$). Furthermore, the gastric damage induced by naproxen was abolished when this drug was combined with DHA. These data suggest that oral administration of DHA-naproxen combination induces gastric safety and supra-additive antinociceptive effect in the formalin test so that this combination could be useful to management of inflammatory pain. Drug Dev Res 78 : 332-339, 2017.

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Key words: naproxen; docosahexaenoic acid; supra-additive interaction; gastric injury; antinociception

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) and opioid analgesics are the two most common classes of drugs used for pain management [Fornasari, 2012]. However, the prescribing of these drugs is accompanied of significant adverse effects; for example, traditional NSAIDs-induce gastric damage and bleeding [Seo et al., 2012] while opioids are frequently accompanied by bradycardia, respiratory depression, physical dependence, sedation, and tolerance [Foroud and Vesal, 2015].

Given the difficulty of finding an effective drug with minimal side effects for the treatment of pain, researchers have evaluated other pharmacological strategies, for example, drug combinations, that allowing the use of lower doses of each drug to improve their therapeutic effect without enhancing side effects [Arroyo-Lira et al., 2014; Macedo et al., 2016]. The combination of NSAIDs with natural products is an alternative to achieve these goals [Maroon et al., 2010]. Thus, interactions between diclofenac and *Matricaria chamomilla* L. or curcumin have been characterized as supra-additive in nociceptive murine models [De Paz-Campos et al., 2014; Ortiz et al., 2016]. Similarly, additive systemic antinociception and gastric safety of a citral-naproxen combination has been demonstrated using an isobolographic analysis [Ortiz et al., 2010].

Naproxen exhibits analgesic, antipyretic, and anti-inflammatory activity and is widely used in the treatment of rheumatic diseases, degenerative joint diseases of the hip and knee, acute gout, dysmenorrhea, or pain following surgery or trauma [Duggan et al., 2010]. This drug, like other nonselective NSAIDs, inhibits prostaglandin synthesis by the nonselective inhibition of the cyclooxygenases (COXs) and can lead to a range of undesirable and sometimes fatal short-and long-term organ toxicities, including gastrointestinal ulceration, bleeding, and toxic effects on liver and kidney [Angiolillo and Weisman, 2016]. Recent evidence suggests that naproxen does not exert cardiovascular side effects in comparison to other NSAIDs [Duggan et al., 2010; Angiolillo and Weisman, 2016].

Docosahexaenoic acid (DHA), an omega-3 long-chain polyunsaturated fatty acid (*n*-3 PUFA), has shown protective effects in ischemia and spinal cord injury and has anti-inflammatory, neuroprotective, cardioprotective, and gastroprotective effects [Belayev et al., 2011; Pineda-Peña et al., 2012; Figueroa et al., 2012, 2013]. DHA also has antinociceptive effects in mouse models of thermal and chemical pain [Nakamoto et al., 2010; Landa-Juárez et al., 2016]. A DHA-indomethacin combination shows a supra-additive antinociceptive effect with improved gastric safety [Arroyo-Lira et al., 2014].

The aims of the present study were to evaluate the effects of DHA on the nociception and pharmacokinetics of naproxen in rats, as well as assessing gastric safety as compared with naproxen alone.

MATERIAL AND METHODS

Animals

Female Wistar rats (7–9 weeks; 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 euthanized in a CO_2 chamber at the end of the assay. All experiments followed the Guidelines on Ethical Standards for Investigation using Animals [Zimmermann, 1983], and the protocol was approved by the Institutional Animal Care and Use Committee CINVESTAV, IPN, Ciudad de México, Mexico.

Drugs

DHA (D2534) and naproxen sodium were purchased from Sigma-Aldrich (Toluca, Mexico). Formaldehyde was purchased from J.T. Baker. The vehicle used for DHA was olive oil. Naproxen was dissolved in 0.9% saline solution.

PHARMACODYNAMIC STUDY AND GASTRIC SECURITY

Measurement of Antinociceptive Activity

Nociception and antinociception were assessed using a formalin test [De Paz-Campos et al., 2014]. Briefly, 50 μL of 1% formalin were injected subcutaneously (s.c.) into the plantar surface of the right hind paw, and the incidence of spontaneous flinching behavior quantified during 1 min every 5 min for a period of 60 min after injection. The data collected between 0 and 10 min post-formalin injection represents the first phase, and the data collected between 15 and 60 min represents the second phase.

Study Design

To determine the antinociceptive effect, at different time regimens before injecting the 1% formaldehyde solution, vehicles or different doses of the tested formulations were administered orally: DHA (56.23, 100, 177.83, and 316.23 mg/kg, 14 h before the formalin insult); naproxen (10, 30, 100, and 300 mg/kg, 1 h before the formalin insult); or the DHA-naproxen combination in the respective time schedules at a fixed ratio-combination of 1:3 based on fractions (1/2, 1/4, 1/8, and 1/16) of their effective dose $(ED)_{30}$ values (6.80, 13.61, 27.21, and 54.43 mg/kg). The investigator performing the experiments was unaware of the treatments that the rats received. For all the experiments, the drug doses and administration time schedules used were selected based on previous reports [Ortiz et al., 2010; Arroyo-Lira et al., 2014; Landa-Juárez et al., 2016] and on pilot experiments in our laboratory.

Data Analysis

The area under the curve (AUC) of the systemic antinociceptive effects produced by each individual and combined drug regimen was calculated as described [De Paz-Campos et al., 2014]. The percent of antinociception for each phase was calculated according to the following equation:

$$\text{Percent of antinociception} = \left[\left(AUC_{\text{vehicle}} - AUC_{\text{post compound}}\right)/AUC_{\text{vehicle}}\right] \times 100$$

Dose response curves were constructed using least-squares linear regression, and the ED_{30} for systemic antinociception induced by DHA and naproxen were calculated according to Tallarida [2000]. The interactions between DHA and naproxen (1:3) was characterized via an isobolographic analysis assuming that the combination comprised equi-effective doses of the individual component drugs. The theoretical additive dose (*Zadd*) and their S.E.M. for the combination in the same component ratio (1:3) was computed from the doses resulting in 30% of the effect (ED_{30}) of the single drug according to the method described by Tallarida [2000] using the following equation: $Zadd = fA + (1 - f)B$, where A is the ED_{30} of DHA, and B is the ED_{30} of naproxen. For a fixed-ratio of 1:3, the value of f is 0.25, and $(1 - f)$ is 0.75. The experimental ED_{30} (*Zexp*) value (and their 95% confidence limit) was determined from the respective drug-dose effect curve of the drug combination according to a standard linear regression analysis of the log dose-response curve [Tallarida, 2000], and the 95% confidence limits were transformed into S.E.M. To construct the experimental antinociceptive effect-dose curve, each group of rats received one of the drugs at the dose used in the Table 1.

TABLE 1. Dosing Amount of Each Drug in the Combination

Combination 1:3 DHA:naproxen (mg/kg, p.o.)		
DHA	Naproxen	Total
4.21	2.59	**6.80**
8.43	5.18	**13.61**
16.85	10.31	**27.21**
33.71	20.72	**54.43**

Gastric Damage

Three hours after administration of naproxen all rats (regardless of treatment) were euthanized in a CO_2 chamber. The stomach was removed and opened along the greater curvature. An observer, blinded to the experimental treatment status of the animals, measured the area (mm^2) of each gastric lesion in the corpus of the stomach using the Image J software [Pineda-Peña et al., 2012].

PHARMACOKINETIC STUDY

Blood Sampling

Rats were lightly anesthetized with ethyl ether and a cannula was surgically implanted into the caudal artery. Animals were divided into two groups of nine rats. One group received an oral dose of 30 mg/kg of naproxen, while another group received the combination fixed dose ratio 1:3 (30/48.81 mg/kg, naproxen/DHA). The systemic doses selected were able to produce antinociception as demonstrated in this study. Whole-blood samples (200 μL) were obtained at 0, 5, 10, 15, 30, 45, 60, 120, 180, 240, 360, 480, and 600 min. DHA was administered 14 h before the naproxen administration.

Naproxen Determination

Whole-blood levels of naproxen were determined by HPLC. Briefly, 100 μL of plasma samples were placed in 1.5 mL Eppendorf tubes, and spiked with 30 μg/mL of diclofenac as an internal standard (100 μL). Proteins were then precipitated by the addition of 800 μL of methanol (final total volume in the tube was 1000 μL). The samples were then vortexed at maximal speed for 3 min and centrifuge at 12,000 rpm for 10 min and the supernatant transferred to a clean tube and 60 μL aliquots were injected into the chromatographic system (Hitachi

Primaide), which consisted of an isocratic pump model Primaide 1110, column oven model 1310, and UV-visible spectroscopy detector Primaide 1410 UV.

Elution of the compound was performed on a 150 mm length, 4.6 diameter C_{18} column of 5 μm particle size (Agilent Eclipse XDB) using a mixture of methanol with water (68:32, v/v; adjusted to pH 3.3) as a mobile phase, and a flow rate of 2 mL/min. Effluent from the column was detected by absorbance at 254 nm. Validation of the analytical method for quantified naproxen was carried out following the Mexican regulatory guidelines (NOM-177-SSA1-2013; procedures and tests to show that a drug is interchangeable) [COFEPRIS, 2013; De Paz-Campos et al., 2014].

Pharmacokinetic Analysis

Retention times were 2.3 and 5 min for naproxen and diclofenac, respectively. Standard calibration curves were constructed in the interval of 0.3–15 μg/mL. A linear relationship ($r = 0.993$) was obtained when AUC ratios of naproxen to the internal standard were plotted against naproxen blood concentration. The selectivity of the method was determined with six different blood blank rats to determine if some compounds were interfering at the retention time of naproxen. Quality control points at low, medium, and high levels (0.9, 6.5, and 12.5 μg/mL, respectively) were used to determine intraday and interday accuracy and precision; coefficients of variation were always lower than 15%, whereas accuracy ranged from 90% to 100%. Pharmacokinetics parameters (C_{max}, T_{max}, $AUC_{0\text{-}t}$, and $t_{1/2}$) were determined by noncompartmental analysis [Patiño-Camacho et al., 2013] using PKSolver [Zhang et al., 2010].

Statistical Analysis

All data are expressed as the means ± S.E.M ($n = 5 - 9$). The dose-response data were analyzed by one-way analysis of variance (ANOVA) using the Newman–Keuls test for the post hoc comparisons. The statistical comparisons between the *Zadd* and *Zexp* values was performed using Student's *t* test according to procedures previously described by Tallarida [2000]. *Zexp* values that were lower than the *Zadd* value, with differences with $P < 0.05$ in both the *X* and *Y* directions, were interpreted as significant *supra-additive* interactions. Values of *Zexp* that were higher than *Zadd* values, with differences with $P < 0.05$ in both the *X* and *Y* directions, were interpreted as significant *subadditive* interactions. The absence of a significant difference between the *Zexp* and *Zadd* values was interpreted as no interaction, and an additive relationship (*additivity*) was thus established in the combination [Tallarida, 2000]. To obtain the interaction index, a fractional analysis was performed using the ED_{30} values of DHA, naproxen, and their combination as described by Tallarida [2002]. Comparison between naproxen bioavailability parameters was carried out by the Student's *t*-test and *P* value of <0.05 was considered statistically significant.

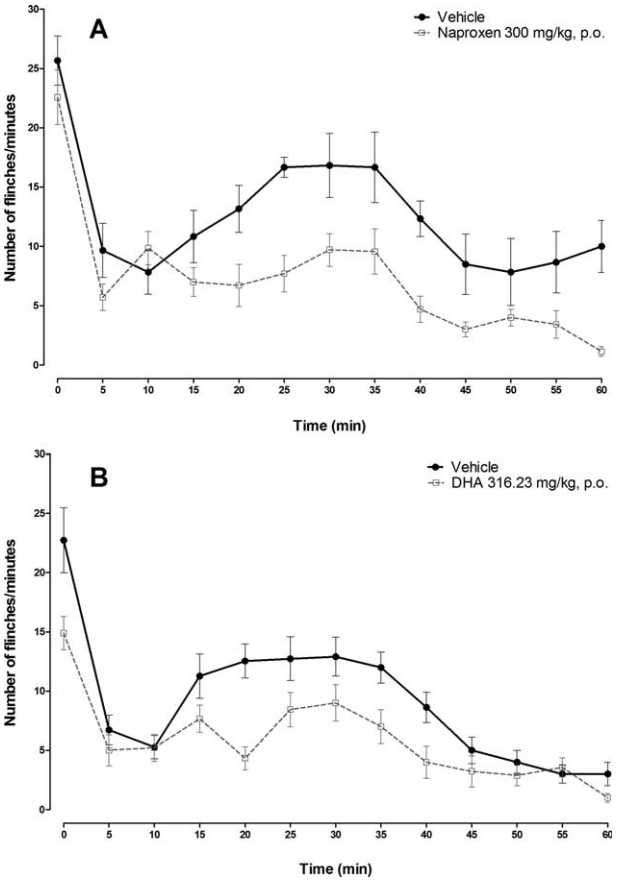
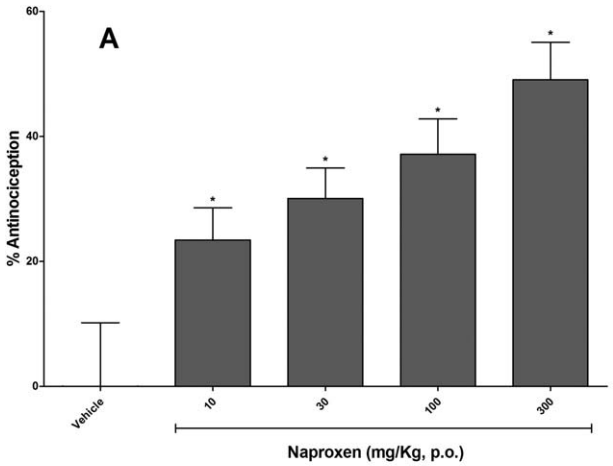


Fig. 1. Time course of the number of flinches per minutes in rats treated with vehicle, (**A**) naproxen (300 mg/kg, p.o.), (**B**) DHA (316.23 mg/kg, p.o.) in the formalin test in the paw of the rat. Data are presented as mean ± S.E.M. ($n = 5 - 9$) $^*P \leq 0.05$ versus respective vehicle (olive oil for DHA and saline for naproxen).

RESULTS

Systemic Antinociceptive Effects Produced by DHA and Naproxen

Administration of formalin (1%) into the plantar surface of the right hind paw produced a typical pattern of flinching behavior characterized by a biphasic time course (Fig. 1). DHA or naproxen given orally produced dose-related antinociceptive effects in the second phase of the formalin test (Fig. 2). As isobolographic analyses are used when both agents are active, only the data from the second phase were subjected to



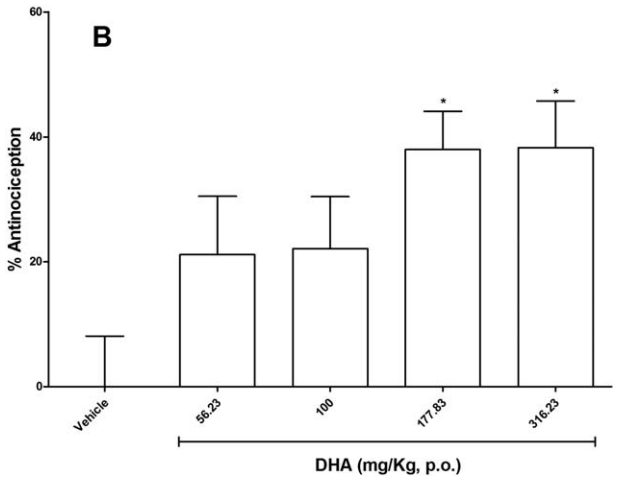


Fig. 2. Dose response curve of the antinociceptive effect of DHA and naproxen on the second phase in the 1% formalin test in the paw of the rat. (**A**) Rats were treated with naproxen at 10, 30, 100, and 300 mg/kg, p.o. (**B**) Rats were treated with DHA at 56.23, 100, 177.83, and 316.23 mg/kg, p.o. Data are presented as mean ± SEM. ($n = 5 - 9$) *$P \leq 0.05$ versus respective vehicle (olive oil for DHA and saline solution for naproxen).

further analysis. The ED_{30} values for systemic DHA and naproxen administration were 134.84 ± 27.72 mg/kg and 27.62 ± 3.80 mg/kg, respectively.

Interactions between DHA and Naproxen

DHA-naproxen combinations given orally at fixed-ratios of 1:3 produced significant dose-dependent antinociception ($P < 0.05$; Fig. 3). The *Zexp* value for the combinations was 33.44 ± 1.90 mg/kg which was lower ($P < 0.05$) than the *Zadd* 54.43 ± 7.49 mg/kg. Fractional analysis of the combination showed that the a/A + b/B interaction index was less than 1.0 (0.61; $P < 0.05$), indicating a supra-additive or synergistic interaction for the combinations (Fig. 4).

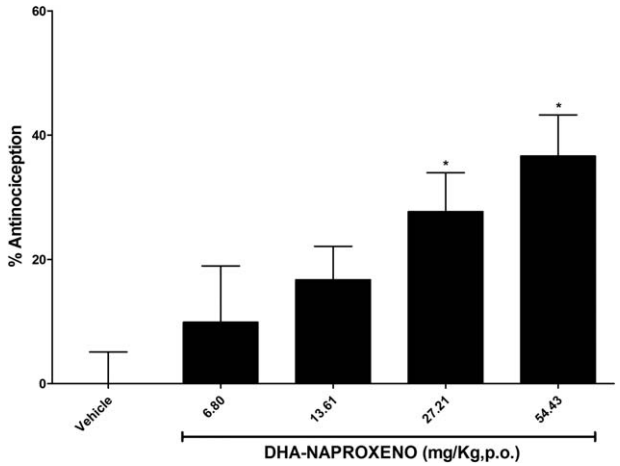


Fig. 3. Dose response curve of the antinociceptive effect of the combination DHA-naproxen on the second phase in the 1% formalin test in the paw of the rat. Data are presented as mean ± S.E.M. ($n = 5 - 9$) *$P \leq 0.05$ versus vehicle.

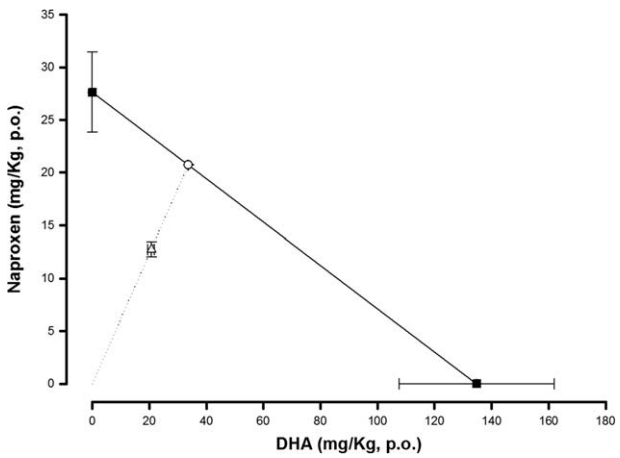


Fig. 4. Isobologram of the combination DHA-naproxen on the second phase in the formalin test. The individual ED_{30} values in each combination (■), the theoretical calculated ED_{30} value for an additive effect (*Zadd*) in a fixed ratio 1:3 (○) and its corresponding experimental ED_{30} values in a fixed ratio 1:3 (*Zexp*, Δ), are represented in the graph. Horizontal and vertical bars indicate S.E.M. The values of *Zexp* were below to *Zadd*, indicating a synergistic relationship for the combinations at fixed-dose ratio 1:3.

Gastric Safety

Oral administration of naproxen, but neither DHA nor the naproxen-DHA combination induced dose-dependent gastric lesions ($P < 0.05$; Table 2).

Pharmacokinetic of Naproxen and the DHA-Naproxen Combination

The pharmacokinetic profile of naproxen is shown in Figure 5. The pharmacokinetic parameters of naproxen with and without DHA are summarized in Table 3. There were no differences in

TABLE 2. Gastric Lesions (mm^2) in the Rat

Drug	Dose (mg/kg, p.o.)	Gastric lesions (mm^2)
Naproxen	**10**	1.4 ± 0.78
	30	8.73 ± 2.43
	100	24.97 ± 5.24*
	300	30.91 ± 5.73*
DHA	**56.23**	0
	100	0
	177.83	0
	316.23	0
Combination		
DHA-naproxen	**6.80**	0
	13.61	0.61 ± 0.54
	27.21	0.52 ± 0.44
	54.43	0.51 ± 0.38

Rats were orally treated with DHA, naproxen or DHA-naproxen combination in 1:3 fixed ratio combination. Data are represented as mean ± S.E.M. n= 5–9.
*$P \leq 0.05$ versus vehicle.

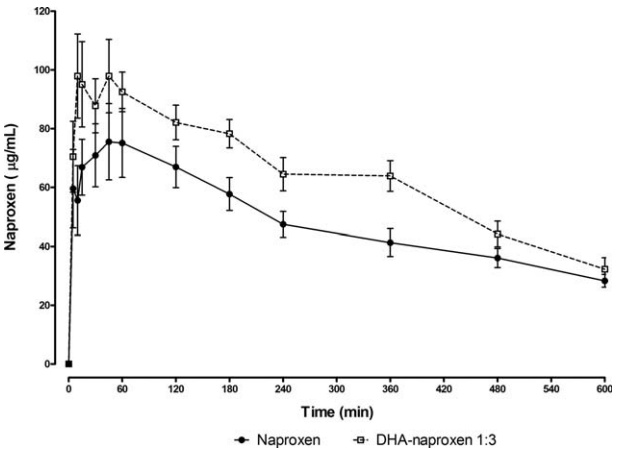


Fig. 5. Mean plasma concentration-time curves in rat after single oral administration of 30 mg/kg naproxen or with 48.81 mg/kg oral dose of DHA. Data are the mean ± S.E.M. of 9 rats.

pharmacokinetic parameters C_{max}, T_{max}, and $t_{1/2}$ of the same doses of naproxen alone or when combined with DHA. Nevertheless, the concomitant administration of DHA increased the $AUC_{0\text{-}t}$ of naproxen ($P < 0.05$; Table 3).

DISCUSSION

Treatment of acute and chronic severe pain remains a major but common challenge faced by clinicians working with the general population. The current study demonstrates that systemic administration of the DHA-naproxen combination (1:3) produces dose-dependent antinociception in the second phase of the formalin test, without gastric injury compared with naproxen alone.

These results demonstrate that the antinociceptive efficacy of DHA, naproxen, and the DHA–naproxen combination treatment is consistent with previous reports that showed the antinociceptive effect of oral naproxen [Ortiz et al., 2010]; and oral and local DHA [Nakamoto et al., 2010; Arroyo-Lira et al., 2014; Landa-Juárez et al., 2016]. Moreover, to the best of our knowledge, this study provides the first demonstration that systemic administration of the DHA-naproxen (1:3) combination possesses supra-additive antinociceptive effects in the formalin test.

Although the mechanisms underlying DHA-naproxen interaction remain unknown, the modification observed in the antinociceptive effect through the isobolographic analysis showed that there is a pharmacodynamic interaction. It has been suggested that a supra-additive interaction can be obtained when two drugs with different and complementary mechanism of actions are associated [De Paz-Campos et al., 2014]. The analgesic properties of naproxen require inhibition of COX involved in the formation of prostaglandins, potent hyperalgesic mediators that modulate multiple sites along the nociceptive pathway and enhance both transduction (peripheral sensitizing effect) and transmission (central sensitizing effect) of nociceptive information. Thus, the inhibition of the formation of prostaglandins at peripheral and central sites by naproxen leads to the normalization of the increased nociception threshold associated with the inflammation induced by the administration of formalin [Burian and Geisslinger, 2005; Duggan et al., 2010].

Conversely, was recently elucidated that the activation of FFA1 receptors by DHA leads to an increased release of β-endorphin from pro-opiomelanocortin neurons, phenomenon that may induce an important role in pain control [Nakamoto et al., 2015]. Additionally, our group recently reported that DHA is able to activate big- and small-conductance Ca^{2+} -activated K^+ channels ($K_{Ca}1.1$, $K_{Ca}2.1$–3) and ATP-sensitive K^+ channels ($K_{ir}6.1$–2) to induce local antinociception on the rat formalin test [Landa-Juárez et al., 2016]. It is possible that besides to the decreasing prostaglandin contents by naproxen, these other mechanisms indicated may contribute to the synergism observed.

Supra-additive interactions may be due to pharmacokinetic interactions. Drug–drug interactions occur during absorption, distribution, biotransformation, and excretion. Absorption is mostly through the intestinal mucosa (duodenum). Drug bioavailability is dependent on an enterocyte P-glycoprotein (P-gp), which actively pumps back drugs into the intestinal lumen, and on enterocyte cytochrome P450 (CYP450) enzymes [Sousa et al., 2008]. Hirunpanich et al. [2008] demonstrated that DHA inhibits

TABLE 3. Pharmacokinetic Parameters of Naproxen After a Single Oral Dose of 30 mg/kg Alone or in the Presence of DHA at 48.81 mg/kg, p.o. in Rat

Treatments	C_{max} (μg/mL)[a]	T_{max} (h)[b]	$AUC_{0\text{-}t}$ (h μg/mL)[c]	$t^1/_2$ (h)[d]
Naproxen	96.18 ± 13.52	0.83 ± 0.24	483.33 ± 43.03	7.56 ± 1.91
Naproxen+DHA	120.60 ± 9.06	0.85 ± 0.29	639.70 ± 29.96*	5.00 ± 1.22

Notes: Data represent as mean ± S.E.M. of nine repetitions for each treatment. * $P \leq 0.05$ versus naproxen.
[a]C_{max} was maximal concentration reached.
[b]T_{max} was determined as the time when the maximal concentration reached.
[c]AUC was determined as area under the concentration versus time curve.
[d]$t^1/_2$ was determined as the time required to eliminate the half of the maximal concentration of naproxen reached.

intestinal CYP3A, but not P-gp, in vivo and in vitro indicating that DHA could be used as a bioavailability enhancer for drugs extensively metabolized by CYP3A in the gut (e.g., cyclosporine and midazolam) [Hirunpanich et al., 2006, 2008].

In the present study, the oral administration of DHA increased the naproxen $AUC_{0\text{-}t}$ suggesting that a modification in the bioavailability of naproxen correlates with the supra-additive antinociceptive effect of the oral DHA-naproxen combination. Since naproxen is metabolized at liver by CYP2A1 and CYP2C9 enzymes [Patiño-Camacho et al., 2013] the real contribution of DHA to inhibit these enzymes requires further elucidation.

Gastric side effects derived of COX inhibition by NSAIDs markedly limit their use [Duggan et al., 2010; Seo et al., 2012]. The suppression of gastric acid secretion with proton pump inhibitors such as omeprazole is a widely used strategy for the management of NSAIDs side effect; nonetheless this drug may exacerbate small intestinal damage [Wallace et al., 2011]. Combinations of NSAIDs with other analgesic agents appear to be an effective strategy to reduce NSAID exposure, allowing the use of lower doses of each agent [Tallarida, 2001; Arroyo-Lira et al., 2014], since it has been reported that gastric toxicity is strongly influenced by the amount of NSAIDs [Seo et al., 2012]. Thus, the combination of NSAIDs with natural products like DHA is an alternative to increase the antinociceptive effects without increasing side effects [Maroon et al., 2010] including gastric injury [Ortiz et al., 2010]. In our study, we showed that oral administration of naproxen induced gastric damage. Additionally, we demonstrated the gastric safety of the oral administration of DHA-naproxen combination. This result is consistent with previous reports where we demonstrated that DHA-induced gastroprotection and gastric safety using DHA-indomethacin combination [Pineda-Peña et al., 2012; Arroyo-Lira et al., 2014].

It is probable that DHA in the DHA-naproxen combination is activating gastroprotective factors and/or inactivating the mechanism responsible for gastric damage induced by naproxen [Pineda-Peña et al., 2012; Arroyo-Lira et al., 2014]. In this particular case, it is probable that the gastroprotection induced by DHA is caused by a reduction of TNF- alpha, interleukin1-beta, or LTB_4 gastric levels [Cho et al., 2011; Pineda-Peña et al., 2012]. However, further studies are required to determine whether the gastroprotective effect of DHA is associated with changes in those inflammatory mediators. The gastrointestinal safety of this combination in human patients during clinical situations also awaits additional validation.

In conclusion, in the present study we demonstrated using an isobolographic analysis that the systemic administration of the DHA-naproxen combination induces gastric safety and a super-additive antinociceptive effect. DHA produced an increase in $AUC_{0\text{-}t}$ of naproxen and it is correlated with the antinociceptive effect of the combination.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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