

Enhanced analgesic effects of nefopam in combination with acetaminophen in rodents

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Abstract. Nefopam, an analgesic drug, effectively elicits antinociception in the majority of noxious and thermal models in rodents. Acetaminophen is among the most commonly used analgesic and antipyretic drugs worldwide, either on prescription or over the counter. The present study aimed to investigate the analgesic activity of nefopam combined with acetaminophen, which was expected to maximize the potency of analgesia and decrease the dose of nefopam required. Three series of experiments, namely acetic acid-induced writhing tests in mice, hot plate tests in mice and tail flick tests in rats, were used to evaluate the analgesic effect. Initially, an optimum proportion of the two drugs, 3.5 mg/kg nefopam (N) + 60 mg/kg acetaminophen (A), was determined by orthogonal array design based on writhing response number. Subsequently, combinations of N and A (1.75 N + 30 A, 3.5 N + 60 A and 7.0 N + 120 A mg/kg) were determined to elicit antinociception in the writhing test ($P < 0.01$ vs. normal saline control) in a dose-dependent manner. In the hot plate test, hot plate latencies up to 60 min after drug treatment were observed. The combination of 7.0 N + 120 A mg/kg exerted a greater cumulative antinociceptive effect throughout the observation period, with an area under the curve value of $1,156.95 \pm 199.30$ area units (AU), compared with that achieved by 7.0 N mg/kg alone (632.12 ± 62.38 AU). Furthermore, both monotherapy and compound therapy exhibited antinociception dose-dependently in the tail-flick test. However, a combination of 5.0 N + 84 A mg/kg exerted greater analgesic effect compared with 5.0 N mg/kg alone. The data obtained demonstrate that acetaminophen may enhance

the antinociceptive activity of nefopam. Thus, coadministration of nefopam with acetaminophen warrants clinical evaluation.

Introduction

Nefopam is a novel type of analgesic drug that exerts non-narcotic effects (1), and thus differs from typical opioids and anti-inflammatory drugs (2). Nevertheless, it effectively elicits antinociception in the majority of noxious and thermal models in rodents (3,4). The mechanisms of action of nefopam may involve inhibition of monoamine reuptake in the central nervous system and glutamatergic pathway, in which calcium channels serve a role (5,6). Certain adverse reactions of nefopam, including dizziness, headache, nausea, vomiting and sweating, have been associated with its central mechanisms (7).

Non-steroid anti-inflammatory drugs (NSAIDs) are clinically used in the management of chronic, inflammatory and postoperative pains. They may inhibit cyclooxygenase (COX) enzyme that catalyzes the conversion of arachidonic acid to generate prostaglandins (PGs) (8-10). PGs are established to serve a role in pain and inflammatory processes (11). Acetaminophen is among the most commonly used analgesic and antipyretic drugs worldwide, either on prescription or over the counter. The drug has generally replaced aspirin and other salicylates in the treatment of mild to moderate pains in conditions without inflammatory involvement, including headache, toothache and dysmenorrhea (12,13). However, the mechanisms of its action remain to be fully defined. The antinociceptive effects of acetaminophen may occur in part through inhibition of the central COX enzymes COX-2 and COX-3 (14).

Results from other studies on the underlying mechanisms have also indicated the involvement of other receptors and transmitters in the central nervous system (15,16). The side effects of NSAIDs most commonly include gastrointestinal problems, hepatotoxicity and nephrotoxicity (17).

In consideration of the proposed individual mechanisms of nefopam and acetaminophen, the present study aimed to investigate the analgesic efficacy of combined therapy with the two drugs, and to determine whether it is possible to reduce the dose of nefopam used. Three animal models were used: The first involved intraperitoneal administration of

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acetic acid (AA) in mice, which simulates acute inflammatory pain in the viscera and induces abdominal writhing; the second and third models involved simulation of acute thermal pains, evaluated through hot plate and tail flick tests. Assessments of these models ultimately aimed to determine the effects of combined drug therapy compared with nefopam alone.

Materials and methods

Animals. The present animal experiments were conducted according to the rules of animal experimentation and the Guide for the Care and Use of Laboratory Animals of Shenyang Pharmaceutical University (Shenyang, China), and the protocol was approved by the Animal Ethics Committee of Shenyang Pharmaceutical University (approval no: SYPU-IACUC-131220-138). The animals used were Kunming mice aged 4-6 weeks old ($n=249$; male and/or female depending on the assay as specified) and Sprague-Dawley male rats aged 6-8 weeks old ($n=77$; sex ratio 1:1), obtained from the Animal Center of Shenyang Pharmaceutical University. The animal weights ranged between 18-25 g for mice and 180-250 g for rats. The animals were group-housed (6 mice per cage) under standard environmental conditions ($22\pm 1^\circ\text{C}$, humidity $60\pm 5\%$, 12 h light/dark cycle) with free access to a standard commercial diet and water ad libitum. After a 7 day adaptation period, all experiments were performed during the light phase.

Drugs. Nefopam hydrochloride and acetaminophen were provided by Shenyang Funing Pharmaceuticals Co., Ltd. (Shenyang, China). They were dissolved in 0.9% sterile saline (normal saline, N.S.; Shenyang Zhiying Pharmaceutical Co., Ltd., Shenyang, China). Nefopam and acetaminophen were intravenously (i.v.) administered at a constant volume of 10 ml/kg body mass in the abdominal constriction and hot plate tests, while the drugs were intraperitoneally (i.p.) injected (10 ml/kg) in the rat tail flick test to prevent tail pain from i.v. injection.

Determination of optimal drug combination through orthogonal design. The orthogonal array method was used to optimize the proportions of nefopam and acetaminophen (18-20). In brief, the L₉ (3²) factorial design, a factorial arrangement with two factors at three levels, was used to indicate the optimal proportions of nefopam and acetaminophen in combination (Table I). The detailed experimental design is presented in Table II.

The analgesic activity of each combination was evaluated through a preliminary abdominal constriction assay in mice (21). Mice were randomized into 9 groups ($n=10$, sex ratio 1:1) and were respectively administered the combinations of nefopam and acetaminophen (i.v.) according to the design in Tables I and II. After 30 min, each mouse was injected (i.p.) with 0.8% AA and was placed in an individual plastic cage for observation. The number of writhing responses in a 20 min observation period was counted by an investigator blinded to the experimental groups, beginning 3 min after administration of the AA solution. A writhing response was characterized as a wave of contraction of the abdominal musculature followed by extension of the hind limbs.

Table I. Orthogonal design for combination treatment.

Level	Factor (mg/kg)	
	Nefopam	Acetaminophen
1	1.5	60
2	2.5	95
3	3.5	130

Abdominal constriction assay in mice. Subsequently, persistent chemical pain was assessed by abdominal constriction assay in mice. In the present test, male and female mice (sex ratio 1:1) were used, randomized into 8 groups. The mice were respectively administered (i.v.) 0.9% N.S. ($n=10$), nefopam (3.5 and 7.0 mg/kg; $n=9$ and 10), acetaminophen (60 and 120 mg/kg; $n=9$ and 10) or combinations of nefopam (N) and acetaminophen (A) (1.75 N + 30 A, 3.5 N + 60 A, 7.0 N + 120 A mg/kg; $n=8$, 10 and 8) 30 min prior to the abdominal constriction test. Each mouse was injected (i.p.) with 0.8% AA at the start of the test and was placed in an individual plastic cage for observation. The number of writhing responses in a 20 min observation period was counted as described above.

Hot plate test in mice. An RB-200 intelligent hot plate apparatus (Chengdu Taimeng Science And Technology Co., Ltd., Chengdu, China) was used. The temperature of the hot plate was set to $55\pm 0.5^\circ\text{C}$ and latency was defined as the period from the time when the animal was placed on the hot plate surface to the time when the animal licked its back paw or jumped off to avoid thermal pain. Female mice were used. The mice with latency of 5-30 sec by duplicated detection were selected to be used in the formal experiment (21). The mice were randomized into 9 groups and were respectively administered (i.v.) 0.9% N.S. ($n=9$), nefopam (3.5 and 7.0 mg/kg; $n=9$ per group), acetaminophen (60, 120 and 240 mg/kg; $n=10$, 10 and 9) or combinations of N and A (1.75 N + 30 A, 3.5 N + 60 A, 7.0 N + 120 A mg/kg; $n=9$, 10 and 10). The latency of hind paw licking was measured at 15, 30, 45 and 60 min after the test compounds were administered. A maximal latency of 60 sec was used to avoid damage to animal skin tissues for any mice exhibiting thermal pain endurance.

Tail flick test in rats. A tail flick assay in rats was performed as described in our previous study (21), for which an automated tail flick test device (SW-200 Analgesia Meter; Chengdu Taimeng Science And Technology Co., Ltd.) was used. Rat tails (distal 1/3rd) were painted with black ink to enhance absorption of heat radiation. An adjustable heat source was directly placed under the tail. The assay is based on the animal flicking its tail away to avoid pain induced by the source of heat. Tail flick latencies required for the rat to remove its tail were determined in sec as an index of nociceptive threshold (22). Pain threshold elongation indicated the analgesic effect of the drug. The rats with latency of 3-20 sec by duplicated detection were selected to be used in the formal experiment. They were randomized into 9 groups and were respectively administered (i.p.) 0.9% N.S. ($n=9$), nefopam

Table II. Experimental design based on L9 (3²) orthogonal array and experimental results.

Run	Independent variable (factors)		Number of writhing responses, mean \pm SEM/20 min
	Nefopam	Acetaminophen	
1	1	1	11.5 \pm 3.3
2	1	2	10.4 \pm 2.0
3	1	3	5.7 \pm 1.8
4	2	1	4.5 \pm 1.6
5	2	2	8.4 \pm 2.1
6	2	3	9.4 \pm 2.3
7	3	1	2.9 \pm 1.1
8	3	2	7.3 \pm 2.4
9	3	3	4.8 \pm 1.4
K ₁	27.50	18.87	
K ₂	22.25	26.04	
K ₃	14.91	19.75	
R	12.59	7.17	

K_i ($i = 1, 2, 3$) represented the sum of writhing responses for each drug at each level. The factor with higher R value was considered to have greater impact on the analgesic effect. R, Kmax-Kmin; SEM, standard error of the mean.

(2.5 and 5 mg/kg; n=9 per group), acetaminophen (42, 84 and 168 mg/kg; n=8 per group), or combinations of N and A (1.25 N + 21 A, 2.5 N + 42 A, 5.0 N +84 A mg/kg; n=9,9 and 8) prior to their subjection to the tail flick test. The latency of tail flicking was determined at 15, 30, 45, 60 and 75 min after drug administration. The maximal latency was limited to 30 sec to protect tail tissues from damage in instances of thermal pain endurance.

Statistical analysis. Results from the groups were calculated as the mean \pm standard error of mean. Statistical analysis was performed with one-way analysis of variance and post hoc multiple comparison between groups was performed with Fisher's protected least significant difference test to compare the data, which were analyzed with SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Cumulative antinociceptive effect during the whole observation period was determined according to the area under the curve (AUC) of the temporal course to compare the complete antinociceptive effects exerted by the tested drugs, alone or in combination. The value of AUC was calculated with SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA, USA).

Results

Optimization of the proportion of nefopam and acetaminophen. Three dose levels of individual doses of nefopam and acetaminophen were represented by 1, 2 and 3, respectively (Table I). The number of writhing responses was determined to evaluate the analgesic effect of each combination. K_i ($i = 1, 2, 3$) represented the sum of writhing responses for nefopam or acetaminophen at each level. Considering that a reduced number of writhing responses indicated a higher analgesic effect, the lower the value of K_i , the greater the analgesic

effect. The drug with higher R value was considered to have a greater impact on the analgesic effect. As depicted in Table II, nefopam was the main factor influencing the analgesic effect of combination therapy. The optimal formula for nefopam was the third level (3.5 mg/kg) and for acetaminophen the first level (60 mg/kg). Based on the results under orthogonal design conditions, the optimum proportions of the two drugs were indicated to be 3.5 mg/kg nefopam plus 60 mg/kg acetaminophen (Table II).

Antinociceptive effect on writhing response in mice. The combinations of 1.75 N + 30 A, 3.5 N + 60 A and 7.0 N + 120 A mg/kg evoked antinociception dose-dependently in the abdominal constriction assay (Fig. 1); writhing response number in each group was 21.4 \pm 2.26, 6.0 \pm 1.05 and 1.0 \pm 0.50, respectively. Acetaminophen used alone at a dose of 60 mg/kg elicited significant antinociception, with writhing number reduced to 25.1 \pm 1.49 during the 20 min period ($P < 0.05$), while 3.5 mg/kg nefopam reduced writhing number to 16.1 \pm 1.61 ($P < 0.01$), compared with 0.9% N.S. control (36.7 \pm 2.58). Co-injection of 3.5 mg/kg nefopam plus 60 mg/kg antinociception (combination 3.5 N + 60 A mg/kg) significantly reduced the number of writhing responses by 63% (to 6.0 \pm 1.05) compared with that of 3.5 mg/kg nefopam alone ($P < 0.01$). High-dose nefopam (7.0 mg/kg) exhibited significant analgesic efficacy with a writhing response number of 3.0 \pm 0.58, as did the high-dose combination treatment (7.0 N+120 A mg/kg; 1.0 \pm 0.5), relative to N.S. control ($P < 0.01$). The high-dose combination decreased the number of writhing responses by 66% compared with high-dose nefopam alone, though there was no significant difference between the groups. Additionally, there was no significant difference in analgesic effect between the optimum combination (3.5 N + 60 A mg/kg) and high-dose nefopam treatments (Fig. 1).

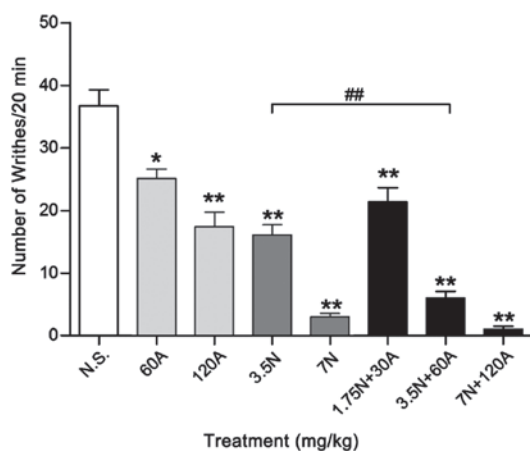


Figure 1. Antinociceptive effects (number of writhing responses) during the acetic acid-induced writhing test in mice. The number of writhing responses were recorded in mice following treatment with different doses of A alone (60 and 120 mg/kg, i.v.), N alone (3.5 and 7 mg/kg, i.v.) or N in combination with A (1.75 N + 30 A, 3.5 N + 60 A and 7 N + 120 A mg/kg, i.v.). Data are expressed as the mean \pm standard error of the mean, $n=8-10$ per group. * $P<0.05$ and ** $P<0.01$ vs. N.S. control; ## $P<0.01$ as indicated. A, acetaminophen; N, nefopam; N.S., normal saline; i.v., intravenous.

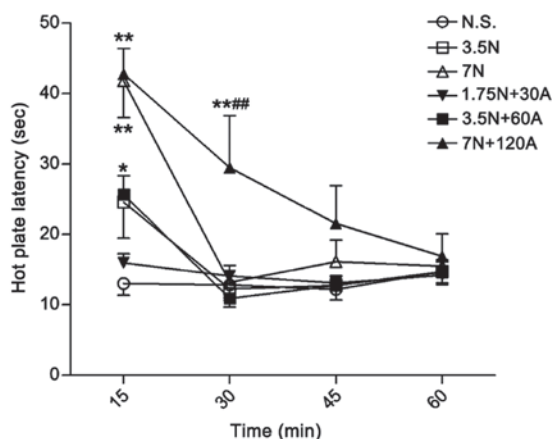


Figure 3. Antinociceptive effect of N alone and in combination in the mouse hot plate test. Paw withdrawal latency in mice was recorded following treatment with different doses of N alone (3.5 and 7 mg/kg, i.v.) and in combination with A (1.75 N + 30 A, 3.5 N + 60 A and 7 N + 120 A mg/kg, i.v.). Latency was assessed at 15, 30, 45 and 60 min after drug administration. Data are expressed as the mean \pm standard error of the mean, $n=9-10$ per group. * $P<0.05$ and ** $P<0.01$ vs. N.S. control at the same time point; ## $P<0.01$ vs. 7 N group at 30 min. A, acetaminophen; N, nefopam; N.S., normal saline; i.v., intravenous.

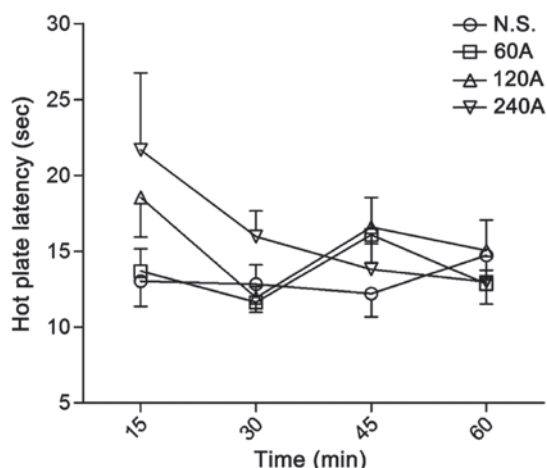


Figure 2. Antinociceptive effect of A in the hot plate test. Antinociceptive effect, based on prolonged paw withdrawal latency in mice, was recorded following treatment with different doses of A (60, 120 and 240 mg/kg, i.v.). Latency was assessed at 15, 30, 45 and 60 min after drug administration. Data are expressed as the mean \pm standard error of the mean, $n=9-10$ per group. No statistically differences were observed. A, acetaminophen; N.S., normal saline; i.v., intravenous.

Antinociceptive effect on hot plate latency in mice.

Acetaminophen alone did not exhibit significant antinociceptive activity in the hot plate assay even at a high dose of 240 mg/kg (Fig. 2), and neither did the low dose of nefopam (3.5 mg/kg; Fig. 3). The combination 3.5 N + 60 A mg/kg exerted a significant antinociceptive effect at 15 min when compared with N.S. control ($P<0.05$; Fig. 3). The analgesic effect of high-dose nefopam (7.0 mg/kg) alone was significant at 15 min when compared with that of the control ($P<0.01$), though was not sustained at time points thereafter (Fig. 3). By contrast, the high-dose combination (7 N + 120 A mg/kg) was identified to produce a significant analgesic effect up to 30 min after administration compared with the control

($P<0.01$; Fig. 3). Furthermore, the high-dose combination exerted greater antinociceptive activity at 30 min compared with high-dose nefopam alone ($P<0.01$; Fig. 3).

The cumulative effect of nefopam or acetaminophen administered individually or in combination, expressed as the AUC of the temporal course, also indicated the different antinociceptive properties of the drugs used alone and in combination (Fig. 4). According to AUC values, acetaminophen at doses of 60, 120 and 240 mg/kg did not exhibit significant antinociceptive activity in the hot plate assay, though exerted dose-dependent effects; the area units (AU) were 397.81 ± 34.81 , 442.54 ± 82.59 and 505.85 ± 58.09 , respectively (Fig. 4). The high-dose combination (7 N + 120 A mg/kg) exhibited a greater cumulative antinociceptive effect during the whole observation period (60 min), with an AUC value of $1,156.95\pm199.30$ AU, compared with that of high-dose nefopam (7 mg/kg) alone (632.12 ± 62.38 AU; $P<0.01$). Nevertheless, both high-dose nefopam and combination exhibited significant cumulative antinociception compared with the N.S. control (381.6 ± 33.9 AU; $P<0.05$ and $P<0.01$, respectively; Fig. 4).

Antinociceptive effect on thermal tail flick latency in rats.

The highest dose of acetaminophen (168 mg/kg) exhibited significant antinociceptive activity in the tail-flick test at 15 min after drug administration ($P<0.05$), while the lower doses (42 and 84 mg/kg) had no significant analgesic effect, relative to N.S. control (Fig. 5). Nefopam alone exerted a sustained analgesic effect at a dose of 5.0 mg/kg, which was significant at 30 ($P<0.01$), 45 ($P<0.01$), 60 ($P<0.05$) and 75 min ($P<0.05$; Fig. 6); while the lower dose of the drug (2.5 mg/kg) only exerted significant analgesic effect at 30 min ($P<0.01$; Fig. 6). By contrast, 42 mg/kg acetaminophen combined with 2.5 mg/kg nefopam (combination 2.5 N + 42 A mg/kg) exerted a significant analgesic effect on tail flick latency compared with the control at all time points ($P<0.05$), particularly between 15-45 min ($P<0.01$; Fig. 6).

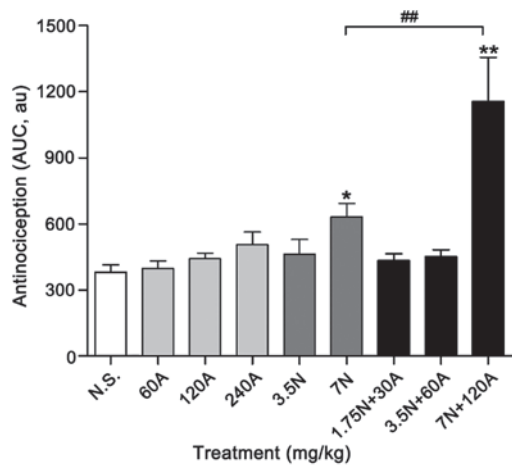


Figure 4. Comparison of the cumulative antinociceptive effect between N alone and N in combination with A in the hot plate test in mice. The cumulative effects were expressed as AUC values (AU). Data are expressed as the mean \pm standard error of the mean, n=9-10 per group. *P<0.05 and **P<0.01 vs. N.S. control at the same time point; ##P<0.01 as indicated. A, acetaminophen; N, nefopam; N.S., normal saline; AUC, area under the curve; AU, area units.

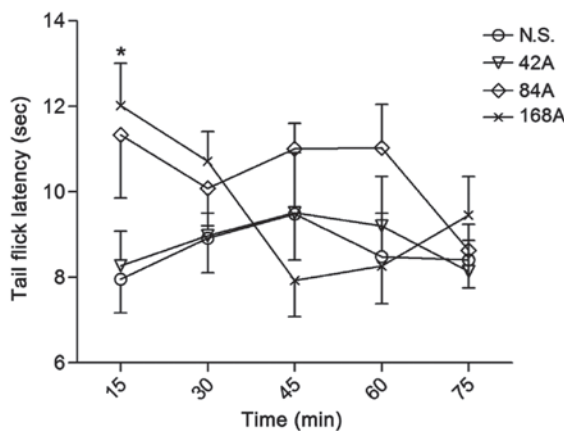


Figure 5. Antinociceptive effect of A in the tail flick test in rats. Tail flick latency in rats was recorded following treatment with different doses of A (42, 84 and 168 mg/kg, i.p.). Latency was assessed at 15, 30, 45, 60 and 75 min after drug administration. Data are expressed as the mean \pm standard error of the mean, n=8-9 per group. *P<0.05 vs. N.S. control at 15 min. A, acetaminophen; N.S., normal saline; i.p., intraperitoneal injection.

This antinociceptive effect was maximized by co-injection of 5 mg/kg nefopam and 84 mg/kg acetaminophen (combination 5 N + 84 A mg/kg) throughout the observation period (P<0.01); additionally, maximum effect of the 5 N + 84 A mg/kg combination was observed at 30 min after drug administration (Fig. 6). The combinations of nefopam with acetaminophen produced greater antinociceptive effect than that obtained by administration of the corresponding nefopam concentration alone, and the analgesic effect of the combination treatments occurred in a dose-dependent manner (Fig. 6). The sustained effects over 75 min were subsequently expressed with AUC values, which verified the assay results (Fig. 7). Notably, both monotherapy and combined therapy exhibited antinociceptive effects in a dose-dependent manner, and the higher dose combination (5 N + 84 A mg/kg)

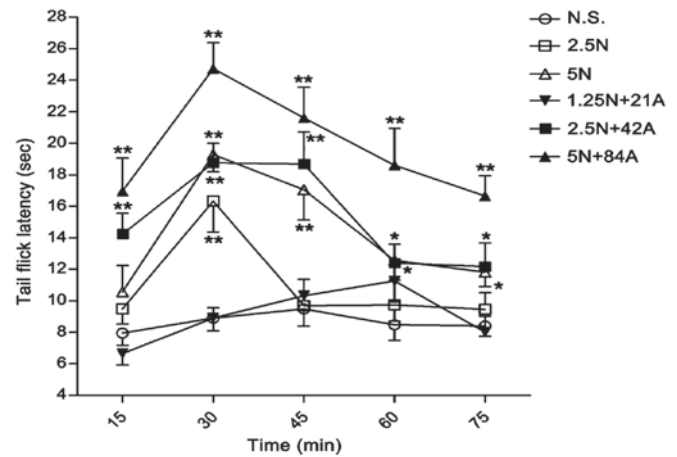


Figure 6. Antinociceptive effect of N alone and in combination in the tail flick test in rats. Tail flick latency in rats was recorded following treatment with different doses of N alone (2.5 and 5 mg/kg, i.p.) and in combination with A (1.25 N + 21 A, 2.5 N + 42 A, and 5 N + 84 A mg/kg, i.p.). Latency was assessed at 15, 30, 45, 60 and 75 min after drug administration. Data are expressed as the mean \pm standard error of the mean, n=8-9 per group. *P<0.05 and **P<0.01 vs. N.S. control at the same time point. A, acetaminophen; N, nefopam; N.S., normal saline; i.p., intraperitoneal injection.

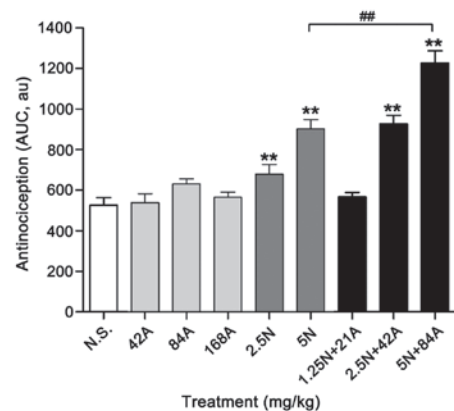


Figure 7. Comparison of the cumulative antinociceptive effect between N alone and N in combination with A in the tail flick test in rats. The cumulative effects were expressed as AUC values (au). Data are expressed as the mean \pm standard error of the mean, n=8-9 per group. **P<0.01 vs. N.S. control; ##P<0.01 as indicated. A, acetaminophen; N, nefopam; N.S., normal saline; AUC, area under the curve; AU, area units.

exerted greater analgesic effect compared with the higher dose of nefopam (5 mg/kg) alone (P<0.05; Fig. 7).

Discussion

The main purpose for the development of combination analgesics is to gain further efficacy and potency, and to thus doses. It has been demonstrated that nefopam has synergistic analgesic effects in humans and rodent animals when administered concomitantly with nonsteroidal anti-inflammatory drugs (3,23-25). A previous clinical trial indicated that a combination of nefopam and ketoprofen produced effective analgesia with synergistic interaction in humans (23). Elstraete and Sitbon (24) reported that the combination of nefopam and acetaminophen produced effective analgesia with a synergistic

interaction allowing a dose reduction of each drug. Furthermore, it has been observed that coadministration of nefopam with ketoprofen or paracetamol has synergistic effects in rats with carrageenan-induced tactile allodynia, in the inflammatory phase of the mouse formalin test, in thermal hyperalgesia in an incision model of postoperative pain, and in the abdominal writhing response in the AA test (3,25). The present study verified an additive potentiation of acetaminophen and nefopam at the tested doses when compared to their per se effect in rodents, and this positive combination allowed dose reduction of each drug.

AA induces a writhing response when it is intraperitoneally administered (26,27). It has been reported that the agent may cause the indirect release of noxious substances including bradykinins, serotonin, histamine and PGs (28-30). The severity of abdominal constriction induced by AA may be dependent on the production and release of pro-inflammatory cytokines from resident peritoneal macrophages and mast cells (31,32). Although the abdominal constriction test is considered a sensitive method for investigating the stimulation of local receptors in the abdomen, it is also considered non-specific as the observed analgesic effects can not be ascertained to involve a peripheral and/or central mechanism (33). In the present study, acetaminophen, nefopam and their combinations produced significant antinociceptive effects in this visceral inflammatory model in mice. The mechanism of action may be dependent on both peripheral and/or central pathways.

Hot plate and tail flick tests were also performed to confirm the additive effect of the two drugs in the present study. The former is often used to investigate the analgesic activity of a drug, which is probably achieved through its effect on central the nervous system (34). Using this test, the present study verified that combined administration of nefopam and acetaminophen has potential as a viable novel therapy for pain. Both methods of treatment, 7 mg/kg nefopam alone and the combination 7 N + 120 A mg/kg, exhibited analgesic effect, though their efficacy differed. Nefopam alone produced a significant analgesic effect at 15 min, while the combination was identified to have greater efficacy for a longer period. Acetaminophen alone did not elicit significant antinociceptive effect even at the highest dose of 240 mg/kg, though markedly enhanced the antinociceptive effect of nefopam when given concomitantly. Furthermore, the higher dose combination (5 N + 84 A mg/kg) in the rat tail flick test exhibited a peak antinociceptive effect at 30 min, and significant antinociception throughout the 75 min observation period compared with the control. Notably, the effect of the higher dose combination in rats was greater than that of nefopam alone at high dose (5 mg/kg; Fig. 7). Gong *et al* (35) identified that a combination of tramadol and acetaminophen produced an additive antinociceptive effect in the tail-flick test. In a previous study by Girard *et al* (3), potent antinociceptive properties of nefopam combined with ketoprofen were observed in a mouse writhing abdominal test. The median effective dose (ED₅₀) of the two drug's coadministration was significantly lowered compared with that of nefopam alone, and it was suggested that the nefopam and ketoprofen interaction may be additive, with a possible tendency toward synergy (3). Another study also illustrated the enhanced antinociceptive properties of combined administration of nefopam and acetaminophen

in a writhing test, in which a 36.3±5.5 mg/kg ED₅₀ value was observed on coadministration compared with 1.5±0.2 and 120.9±14.8 mg/kg for nefopam and acetaminophen, respectively (25). Meanwhile, Miranda *et al* (36) confirmed a synergistic interaction between paracetamol and tramadol on their coadministration via i.p. or intrathecal (i.t.) routes, with the interaction index value of i.p. being similar to that of i.t. It was suggested that the different mechanisms of action of paracetamol and tramadol probably contributed to the analgesic synergism between them (36). In the present study, it was identified that the combination of nefopam and acetaminophen additively reduced pain behavior and increased antinociceptive activity, which is consistent with the results of the previous studies. Additionally, the present results provide novel understanding of the combined application of nefopam and acetaminophen in other rodent pain models besides the mouse writhing model.

In the present study, acetaminophen exhibited weak analgesic action in the mouse hot plate model even at the highest dose of 240 mg/kg and elicited weak antinociceptive effect in the rat tail flick test at 168 mg/kg. Unlike most NSAIDs, acetaminophen administration at therapeutic doses has little or no anti-inflammatory or anti-platelet activity (29). Additionally, it does not exhibit the typical side effect profile of NSAIDs, which includes gastrointestinal tract problems and aspirin-induced asthma (37,38). Although acetaminophen is among the most popular and widely used analgesics, the mechanism of its analgesic action remain uncertain, though the most suggested mechanism involves inhibition of COX activity (39).

The COX-3 isoform of COX is inhibited by acetaminophen at higher affinity than the two other isoforms (COX-1 and -2) (40), though the clinical relevance of this is disputed (41). Serotonin (also known as 5-hydroxytryptamine, 5-HT) exerts effects via several subtypes of its cognate receptor, and its localization in axon termini places these subtypes in prime location for the modulation of pain, transmission and processing (42,43). Previous studies have reported that acetaminophen may exert antinociceptive effect through increasing the levels of serotonin released from serotonergic neurons in the brainstem, known as central mediation (42,43). Furthermore, results have suggested that acetaminophen acts directly on the opioid receptor or cannabinoid (CB) receptor, or indirectly by increasing levels of the endogenous ligands including opioid or anandamide (44,45). It is documented that the metabolism of acetaminophen in the brain and dorsal root ganglia leads to the generation of N-(4-hydroxyphenyl)-arachidonylamide (AM404), which prohibits the cellular reuptake of CB (44). AM404 may also inhibit purified COX-1 and COX-2, which in turn reduces the generation of prostaglandin in the brain (46). Although acetaminophen alone exhibited little antinociceptive activity in the thermal stimulation model in the present study, the effect of nefopam combined with acetaminophen appeared additive in both the mouse hot plate test and rat tail flick test. Furthermore, the analgesic duration was lengthened when the two drugs were coadministered.

Nefopam, a non-opioid analgesic, is derived from diphenhydramine, a histamine H₁ receptor antagonist. A primary hypothesis on the mechanism of analgesic action of nefopam focuses on the inhibition of monoamine uptake in synapses, which would lead to an increase in noradrenaline, dopamine

and serotonin (25). The analgesic action of nefopam may involve the serotonergic or noradrenergic descending pathways; a previous study demonstrated that depletion of spinal noradrenaline attenuated the analgesic action of i.t. nefopam during phase 1, but not during phase 2, of a formalin test (47). Furthermore, it has been confirmed in an *in vivo* animal pain model that monoamine depletion inhibited nefopam antinociception, which indicated the involvement of neuromediators in nefopam-induced analgesia (6). More recently, a number of studies have been conducted on the receptor subtypes that are involved in nefopam antinociception. Girard *et al* (48) suggested that endogenous histamine may indirectly modulate nefopam antinociception in the mouse writhing and formalin tests to a low extent through histamine H₃ receptors, due to a low affinity of nefopam with histamine H₃ receptors. Other results have illustrated that nefopam antinociception may be blocked by serotonergic 5-HT_{1B} receptor antagonist in the writhing test and serotonergic 5-HT_{2C} receptor antagonist in the formalin test respectively, which indicates that the serotonergic system may mediate nefopam antinociception directly and/or indirectly through the serotonin 5-HT_{1B} and 5-HT_{2C} receptor subtypes (49). Furthermore, the analgesic activity of nefopam was adjusted by adrenergic α_1 and α_2 receptors as well as dopaminergic D₂ receptors (49).

It is evident that the levels of serotonin may be associated with the antinociceptive activity of acetaminophen and nefopam through various mechanisms of action. In terms of their combination in the present study, there was an apparent synergistic analgesic effect. Regarding the potential target sites, it has been indicated that the synergism of nefopam and acetaminophen may occur in connection with the descending serotonergic (5-HT) pathways and spinal serotonin 5-HT receptors. It has been reported that the potential effect of acetaminophen is indirectly/directly related to 5-HT₃ receptors at spinal sites (50,51) and descending serotonergic pathways (52). Similarly, the action of nefopam have been indicated to involve serotonergic pathways and 5-HT receptors (49,53). Therefore, the analgesic activity of the combination may be connected in part with serotonin. However, further investigation into the molecular mechanism of the antinociceptive potentiation between nefopam and acetaminophen should now be performed, and the potential pharmacokinetic interaction between the drugs should be studied in detail.

In conclusion, the data obtained from the present study demonstrated that the combination of the non-opioid compound nefopam with the atypical NSAIDs acetaminophen exerted more potent antinociceptive effects than those of nefopam and acetaminophen alone in mouse and rat models with acute and persistent pains. Notably, the combined therapy of the two drugs appeared to increase the antinociceptive effect. These results may provide pre-clinical support for the application of combination therapy with nefopam and acetaminophen in the management of acute pains.

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