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Antinociceptive effects of *Nigella sativa* oil and its major component, thymoquinone, in mice

Abdel-Fattah Mohamed Abdel-Fattah [a,b], Kinzo Matsumoto [a,*], Hiroshi Watanabe [a]

[a] *Department of Pharmacology, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*
[b] *Department of Pharmacology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt*

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Abstract

The antinociceptive effects of *Nigella sativa* oil and its major component, thymoquinone, were examined in mice. The p.o. administration of *N. sativa* oil (50–400 mg/kg) dose-dependently suppressed the nociceptive response in the hot-plate test, tail-pinch test, acetic acid-induced writhing test and in the early phase of the formalin test. The systemic administration (2.5–10 mg/kg, p.o. and 1–6 mg/kg, i.p.) and the i.c.v. injection (1–4 μg/mouse) of thymoquinone attenuated the nociceptive response in not only the early phase but also the late phase of the formalin test. Naloxone injected s.c. (1 mg/kg) significantly blocked *N. sativa* oil- and thymoquinone-induced antinociception in the early phase of the formalin test. Moreover, the i.c.v. injection of naloxone (10 μg/mouse), the μ_1-opioid receptor antagonist, naloxonazine (1–5 μg/mouse), or the κ-opioid receptor antagonist, nor-binaltorphimine (1–5 μg/mouse), significantly reversed thymoquinone-induced antinociception in the early phase but not the late phase of the formalin test, whereas the δ-opioid receptor antagonist, naltrindole (1–5 ng/mouse, i.c.v.), had no effect on either phase. The antinociceptive effect of morphine was significantly reduced in thymoquinone- and *N. sativa* oil-tolerant mice, but not vice versa. These results suggest that *N. sativa* oil and thymoquinone produce antinociceptive effects through indirect activation of the supraspinal μ_1- and κ-opioid receptor subtypes.

Keywords: *Nigella sativa* oil; Thymoquinone; Antinociception; Opioid system; Formalin test

1. Introduction

Nigella sativa L. seeds (Ranunculaceae) have been used for thousands of years as a spice and food preservative, as well as a protective and curative remedy for numerous disorders (Chopra et al., 1956; Nadkarni, 1976). It has been demonstrated that the crude oil prepared from the seeds and its chemical components produce a variety of pharmacological actions such as anti-histamine (El-Dakhakhny, 1965; Mahfouz et al., 1965; Chakravarty, 1993), anti-hypertensive (El-Tahir et al., 1993), hypoglycemic (Al-Hader et al., 1993), anti-microbial (Hanafy and Hatem, 1991), and immunopotentiating effects (Worthen et al., 1998). In addition, Houghton et al. (1995) have reported that thymoquinone (Fig. 1), one of the components of *N. sativa* L. seed oil, inhibits ascorbic acid-Fe^{3+}-induced peroxidation of brain phospholipids and generation of eicosanoids, thromboxane B_2 and leukotriene B_4. These pharmacological properties appear to be involved in the beneficial effect of *N. sativa* oil on rheumatism and related inflammatory diseases. However, to our knowledge, no information is available on the pharmacological effects of *N. sativa* oil and thymoquinone in the central nervous system. In the present study, to clarify the neuropharmacological profiles of *N. sativa* oil and thymoquinone, we investigated the effects of *N. sativa* oil and thymoquinone on the nociceptive responses of mice to various nociceptive stimuli, and elucidated the possible mechanism(s) underlying the action of *N. sativa* oil and thymoquinone using the formalin test. We also tested the

* Corresponding author. Tel.: +81-76-434-7612; fax: +81-76-434-5056.
E-mail address: mkinzo@ms.toyama-mpu.ac.jp (K. Matsumoto).

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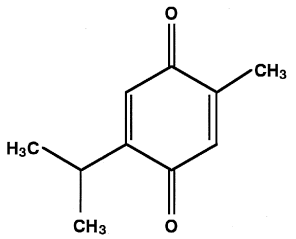

Fig. 1. Chemical structure of thymoquinone.

possible development of tolerance to these agents in response to repeated administration.

2. Materials and methods

2.1. Animal

Male ddY mice weighing 24–32 g (Japan SLC, Hamamatsu, Japan) were used. Mice were housed in groups of 20 per cage (35 × 30 × 16 cm) for at least 1 week before the experiments. Housing conditions were thermostatically maintained at 24 ± 2°C with constant humidity (55 ± 5%) and a light/dark cycle (lights on: 0730–1930). Animals were given food and water ad libitum. The present studies were conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

2.2. Drugs

N. sativa oil was obtained from Pharco Pharmaceuticals, Alexandria, Egypt. The following drugs were used: naloxone HCl, naltrindole HCl and thymoquinone (Sigma, St. Louis, MO); naloxonazine 2HCl (Wako, Osaka, Japan), and nor-binaltorphimine 2HCl (Research Biochemicals, Natick, MA). *N. sativa* oil was suspended in propylene glycol. Thymoquinone was dissolved in saline containing 0.1% Tween 80. Naloxonazine was dissolved in 0.2% acetic acid and the pH was adjusted up to 7.4 with NaOH. The other drugs were dissolved in saline. All drug solutions were prepared just before the start of the experiments. Oral (p.o.) administration, intraperitoneal (i.p.) and subcutaneous (s.c.) injections were performed using a volume of 0.1 ml/10 g body weight and intracerebroventricular (i.c.v.) injection was performed using a volume of 5 μl/mouse according to the method described by Haley and McCormick (1957).

2.3. Antinociceptive activity

2.3.1. Hot-plate test

The hot-plate test was carried out as previously reported (Matsumoto et al., 1996a,b; Thonpradichote et al., 1998). An animal was placed on a metal plate maintained at 55 ± 1°C and the latency of nociceptive responses such as licking or flicking of the hindlimb or jumping was measured according to the method of Hunskaar et al. (1986). Only the mice that showed the nociceptive response within 18 s were used for the experiments. The latency of nociceptive responses in these animals was expressed as the hot-plate latency. A cut-off time of 45 s was selected to prevent tissue damage. Thirty minutes after administration of the test agents, the hot-plate latency was measured every 30 min over a 4-h period.

2.3.2. Writhing test

For testing writhing behavior, 0.6% acetic acid solution (10 ml/kg body weight) was injected i.p., and the number of writhing and stretching movements was counted over a 5-min period as described by Hendershot and Forsaith (1959). Writhing was defined as contraction of the abdominal muscles accompanied by extension of the hind limbs. *N. sativa* oil (50–400 mg/kg, p.o.) was administered 60 min before acetic acid. The percentage inhibition was determined for each experimental group of 10 mice as follows:

$$\%\,\text{inhibition} = 100 \times (1 - \text{No. of writhing in experimental group} / \text{No. of writhing in control group}).$$

2.3.3. Tail-pinch test

The nociceptive response in the tail-pinch test was measured according to the modified Haffner's method as previously reported (Matsumoto et al., 1996a,b). Briefly, mice were pre-tested by pinching of their tails with hemostatic forceps (3-mm width, 500 g constant pressure), and only the mice that showed nociceptive responses such as biting the forceps or vocalization within 2 s were used for the experiments. The latency of nociceptive responses in these animals was expressed as the tail-pinch latency. To minimize tissue damage, a cut-off time of 6 s was selected. Sixty minutes after p.o. administration of *N. sativa* oil, the nociceptive responses were measured every 15 min over a 45-min period.

2.3.4. Formalin test

The nociceptive response in the formalin test was measured using a slight modification of the method described in our previous report (Reanmongkol et al., 1994). Briefly, each mouse was placed in a transparent plastic cage and left for 5 min before formalin injection to allow habituation to the new environment. Ten microliters of 2% formalin was injected s.c. to the plantar region of the right hind paw of mice. The time spent exhibiting nociceptive responses such as licking and/or biting of the injected paw was recorded during 5-min periods over a 30-min observa-

tion time. The data were expressed as total duration of nociceptive responses in the early phase (0–10 min) and the late phase (10–30 min) after formalin injection as previously described (Takeshita and Yamaguchi, 1998). P.o., i.p. and i.c.v. administration of test drugs was performed 60, 30 and 15 min before formalin injection, respectively.

2.4. Identification of opioid-receptor subtypes in thymoquinone-induced antinociception in the formalin test

To identify the opioid receptor subtypes involved in the antinociceptive action of thymoquinone, the non-selective opioid receptor antagonist, naloxone (10 μg/mouse), the selective μ_1-opioid receptor antagonist, naloxonazine (1–5 μg/mouse), and the selective κ-opioid receptor antagonist, nor-binaltorphimine (1–5 μg/mouse), were injected i.c.v. 15 min, 24 h and 30 min before thymoquinone, respectively. The selective δ-opioid receptor antagonist, naltrindole (1–5 ng/mouse, i.c.v.), was given just before thymoquinone administration. The doses and time courses of opioid receptor antagonist administration were chosen according to previous reports (Vaccarino et al., 1992; Chiba et al., 1996; Thonpradichote et al., 1998; Sato et al., 1999). Thymoquinone was injected i.p. at a dose of 4 mg/kg 30 min before formalin injection.

2.5. Measurement of spontaneous motor activity in mice

An Animex activity meter (MK-110, Muromachi Kikai, Tokyo) was used to measure spontaneous motor activity in mice. Mice were given *N. sativa* oil or vehicle p.o., and then returned to their home cage. Sixty minutes after administration, three mice of each vehicle- or *N. sativa* oil-treated group were placed in a cage (35 × 40 × 35 cm) and their motor activity was measured over a 30-min period. Each group consisted of 21 mice.

2.6. Induction of tolerance to thymoquinone antinociception and cross-tolerance to morphine antinociception

N. sativa oil (400 mg/kg, p.o.), thymoquinone (4 mg/kg, i.p.) or morphine (5 mg/kg, s.c.) was administered daily for 4 days. On the fifth day, the animals were challenged with the same drug to test the development of tolerance, or a different drug to test cross-tolerance to morphine. Antinociceptive activity was measured 30 min after s.c. morphine or i.p. thymoquinone, or 60 min after p.o. *N. sativa* oil.

2.7. Statistical analysis

Data obtained in the formalin test were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons among different groups or Student's *t*-test between two groups. Data obtained in the hot-plate test, tail-pinch and writhing tests were analyzed by the Kruskal–Wallis analysis of variance followed by Dunn's test for multiple comparison among different groups. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Effects of N. sativa oil on the nociceptive responses of mice in the tail-pinch test, hot-plate test, writhing test and formalin test

The p.o. administration of *N. sativa* oil (50–400 mg/kg) significantly increased the latency of nociceptive responses in the hot-plate and tail-pinch tests (Fig. 2) and attenuated the writhing behavior caused by the i.p. injection of 0.6% acetic acid (Table 1) in a dose-dependent

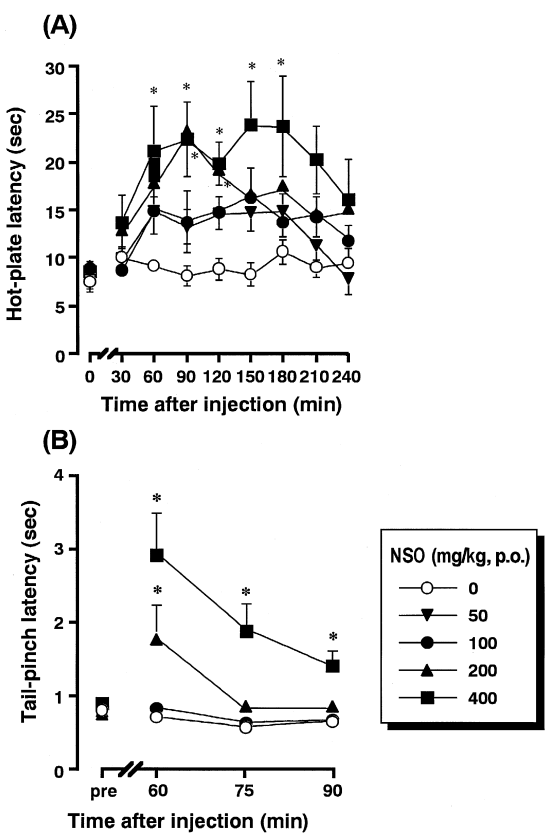

Fig. 2. Effects of *N. sativa* seed oil on nociceptive responses in the hot-plate (A) and tail-pinch tests (B) in mice. Vehicle or *N. sativa* seed oil (NSO) was administered orally 30 and 60 min before the start of the hot-plate and tail-pinch tests, respectively. The latency to nociceptive responses was measured every 30 min in the hot-plate test (A) or every 15 min in the tail-pinch test (B), and expressed as the mean ± S.E.M. Each group consisted of 12–15 mice. * $P < 0.05$ compared to vehicle control.

Table 1
Effect of *N. sativa* oil on acetic acid-induced writhing behavior in mice

Drug	Dose (mg/kg, p.o.)	No. of writhing movements (count/5 min)	% Inhibition
Control		22.2 ± 1.0	
N. sativa oil	50	18.0 ± 0.8	23
	100	14.1 ± 1.1[a]	41
	200	13.4 ± 1.3[a]	46
	400	10.7 ± 1.4[a]	60

After injection of 0.6% acetic acid (10 ml/kg, i.p.), the number of writhing and stretching movements was counted over a 5-min period. *N. sativa* oil (50–400 mg/kg, p.o.) was administered 60 min before acetic acid.
Each value is the mean ± S.E.M for 10 mice.
[a] $P < 0.05$ compared to control.

manner. *N. sativa* oil also significantly suppressed the nociceptive response in the early phase, but did not reduce the response in the late phase of the formalin test (Fig. 3A,B). As shown in Fig. 3C, the effect of *N. sativa* oil in the early phase of the formalin test was significantly blocked by pretreatment with naloxone (1 mg/kg, s.c.).

3.2. Effects of N. sativa oil on spontaneous motor activity in mice

No significant change in the spontaneous motor activity of mice was found following the p.o. administration of *N. sativa* oil (50–400 mg/kg). The spontaneous motor activities of vehicle-, and 100, 200 and 400 mg/kg *N. sativa* oil-treated mice were 1053.4 ± 163.8, 953.3 ± 114.4, 1000.0 ± 113.8, and 822.7 ± 57.1 (the mean counts/30 min/three mice ± S.E.M. from seven independent experiments), respectively.

3.3. Effects of thymoquinone on formalin-induced nociceptive responses in mice

As illustrated in Fig. 4, the systemic administration (2.5–10 mg/kg, p.o. or 1–6 mg/kg, i.p.) and i.c.v. injection (1–4 μg/mouse) of thymoquinone dose-dependently attenuated the nociceptive response in the early and late phases of the formalin test. The s.c. (1 mg/kg) and i.c.v. injection (10 μg/mouse) of naloxone significantly antagonized the effect of systemic thymoquinone (4 mg/kg, i.p.) on the early phase response (Fig. 5A,B). In contrast, s.c. and i.c.v. naloxone failed to reverse thymoquinone-induced antinociception in the late phase.

3.4. Effects of intracerebroventricular injections of naloxone, naloxonazine, naltrindole and nor-binaltorphimine on the antinociceptive activity of i.p. thymoquinone in mice

In order to identify the opioid receptor subtype(s) implicated in the action of thymoquinone, the effects of selective antagonists of opioid receptor subtypes on thymoquinone-induced antinociception were examined in the formalin test. As shown in Fig. 6, i.c.v. injection of the

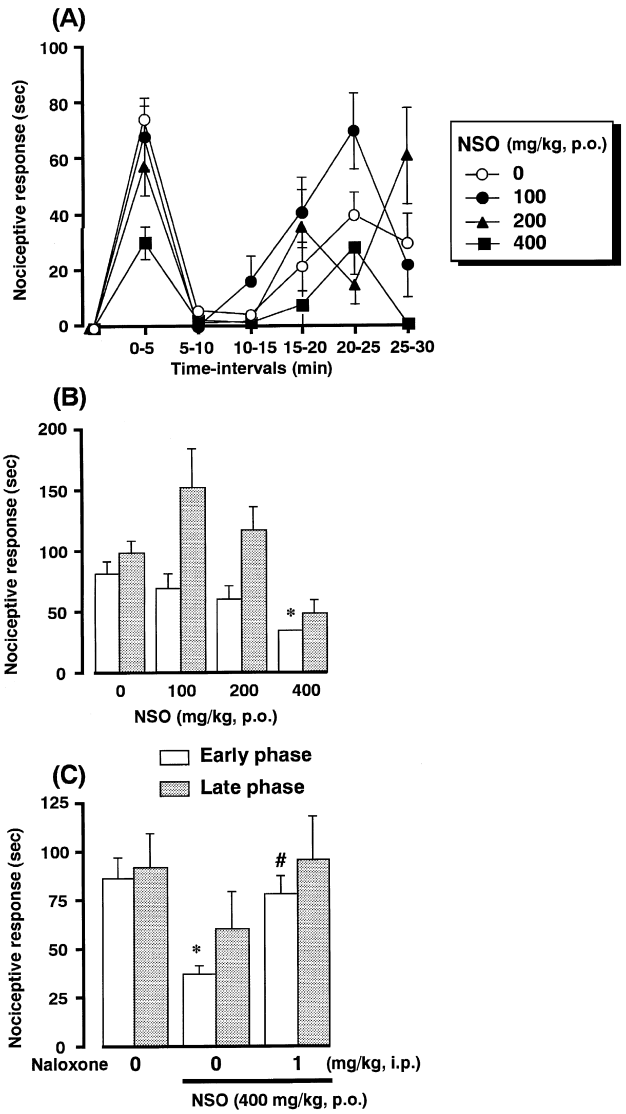

Fig. 3. Effects of *N. sativa* oil on the nociceptive responses in the formalin test. Vehicle or *N. sativa* oil was administered orally 60 min before the start of the experiments. Formalin solution (2%) was injected into the plantar area of the hindlimb at time 0. Time spent biting and/or licking the injected site was recorded during 5-min periods over a 30-min observation time. (A) Time course of *N. sativa* oil (100–400 mg/kg, p.o.)-induced antinociceptive effect in the formalin test. Each data point represents the mean ± S.E.M. for 10–12 mice. (B) Effects of *N. sativa* oil on the early phase and late phase responses in the formalin test. The nociceptive responses in the early phase (0–10 min after formalin injection) and late phase (10–30 min after formalin injection) were recorded. Each column represents the mean ± S.E.M. for 10–12 mice. (C) Effect of naloxone on *N. sativa* oil-induced antinociception in the formalin test. Each column indicates the mean ± S.E.M. Naloxone (1 mg/kg, s.c.) was administered 15 min before *N. sativa* oil administration. * $P < 0.05$ compared to respective vehicle alone. # $P < 0.05$ compared to *N. sativa* oil alone.

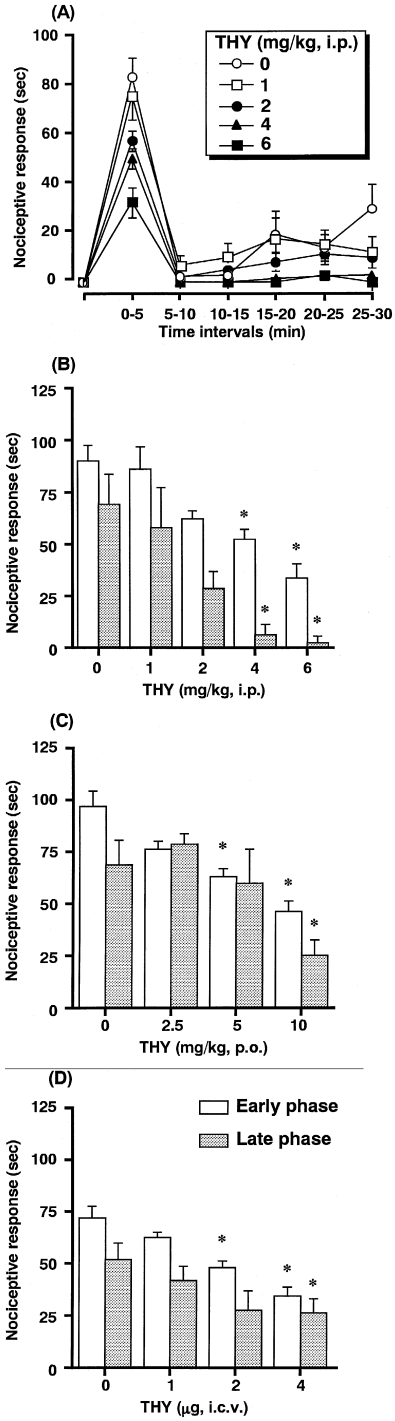

Fig. 4. Effects of thymoquinone, a component of *N. sativa* seed oil, on the nociceptive responses in the formalin test. Vehicle or thymoquinone (THY) was administered i.p. 30 min before the start of the experiments. Formalin solution (2%) was injected into the plantar area of the hindlimb at time 0. Time spent biting and/or licking the injected site was recorded during a 5-min interval throughout a 30-min observation period. (A) Time course of thymoquinone (1–6 mg/kg, i.p.)-induced antinociceptive effect in the formalin test. Each data point represents the mean ± S.E.M. (B, C, and D) Effects of thymoquinone (i.p., p.o. or i.c.v.) on the early phase and late phase responses in the formalin test The nociceptive responses in the early and late phases were recorded as in Fig. 3. Each column represents the mean ± S.E.M. for 10–12 mice. $^{*}P < 0.05$ compared to respective vehicle alone.

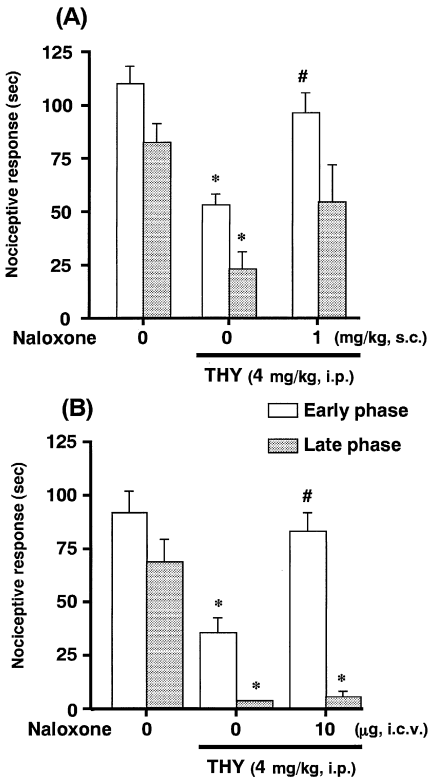

Fig. 5. Effects of s.c. and i.c.v. injections of naloxone on thymoquinone-induced antinociception in the formalin test. Thymoquinone (THY: 4 mg/kg) was administered i.p. 30 min before the injection of 2% formalin into the plantar area of the hindlimb. Naloxone was injected s.c. (1 mg/kg) or i.c.v. (10 μg/mouse) 15 min before thymoquinone administration. The nociceptive responses in the early phase (open columns) and late phase (dotted columns) were recorded as in Fig. 3. Each column represents the mean ± S.E.M. for 10–12 mice. $^{*}P < 0.05$ compared to vehicle alone. $^{\#}P < 0.05$ compared to thymoquinone alone.

μ_1-opioid receptor antagonist, naloxonazine (1 and 5 μg/mouse), or the κ-opioid receptor antagonist, nor-binaltorphimine (1 and 5 μg/mouse), significantly reversed the antinociceptive effects of thymoquinone in the early phase of the formalin test. In contrast, the selective δ-opioid receptor antagonist, naltrindole (1–5 ng/mouse, i.c.v.), had no effect on thymoquinone-induced antinociception. None of the opioid receptor antagonists tested showed any effect on the thymoquinone-induced antinociception in the late phase of the formalin test (Fig. 6).

3.5. Development of tolerance to the antinociceptive effects of N. sativa seed oil and thymoquinone

The development of tolerance to the antinociceptive effects of test drugs is shown in Fig. 7. Compared to the effects observed in naive mice (Fig. 7A, day 1), the antinociceptive effects of morphine (5 mg/kg, s.c.), *N. sativa* oil (400 mg/kg, p.o.), and thymoquinone (4 mg/kg, i.p.) on the early phase response in the formalin test were

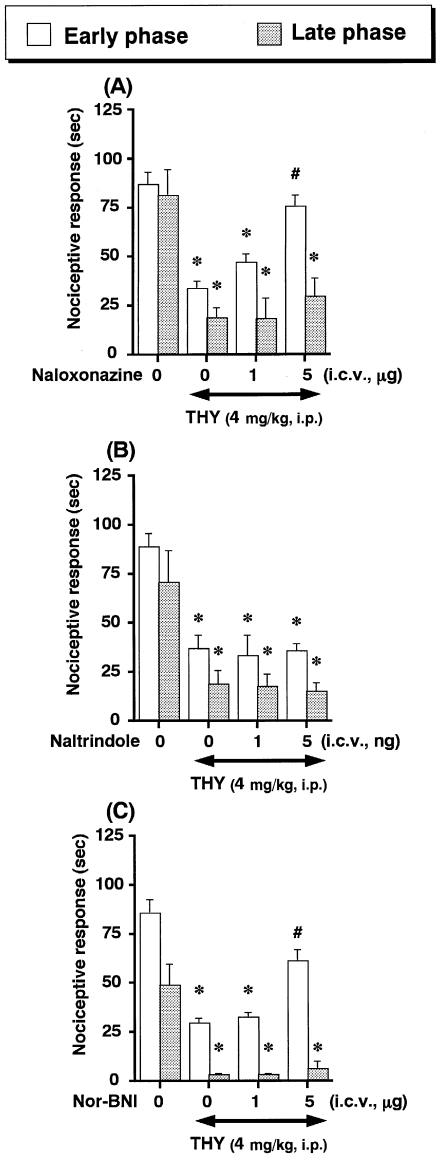

Fig. 6. Effects of i.c.v. injections of naloxonazine, naltrindole and nor-binartorphimine on thymoquinone-induced antinociception in the formalin test. Vehicle or thymoquinone (THY: 4 mg/kg, i.p.) was administered 30 min before 2% formalin injection into the plantar area of the hindlimb. The selective μ_1-opioid receptor antagonist, naloxonazine (A: 1–5 μg/mouse), the selective δ-opioid receptor antagonist, naltrindole (B: 1–5 ng/mouse), or the selective κ-opioid receptor antagonist, nor-binaltorphimine (C: Nor-BNI, 1–5 μg/mouse), was injected i.c.v. 24 h, 0 and 30 min, respectively, before thymoquinone injection. The nociceptive responses in the early and late phases were recorded as in Fig. 3. Each column represents the mean ± S.E.M. for 10–12 mice. $^{*}P < 0.05$ compared to vehicle alone. $^{\#}P < 0.05$ compared to thymoquinone alone.

significantly reduced in the animals that received repeated administration of these drugs (Fig. 7A, day 5). Particularly, *N. sativa* oil- and thymoquinone-induced antinociception in the early phase almost completely disappeared following repeated treatment. The suppressive effects of morphine and thymoquinone on the late phase responses in the formalin test were also significantly attenuated on repeated administration (Fig. 7B). However, in the animals treated with daily administration of thymoquinone, a significant attenuation of the late phase nociceptive response was still observed on challenge with thymoquinone on day 5.

3.6. Cross-tolerance between antinociception produced by morphine and thymoquinone in the early phase response of the formalin test

Cross-tolerance was tested after repeated administration of morphine (5 mg/kg, s.c.), *N. sativa* oil (400 mg/kg, p.o.) or thymoquinone (4 mg/kg, i.p.). When *N. sativa* oil and thymoquinone were given to morphine-tolerant mice on day 5, they produced antinociception without a significant alteration of their effects in this group (Fig. 8). On the other hand, when *N. sativa* oil- and thymoquinone-tolerant

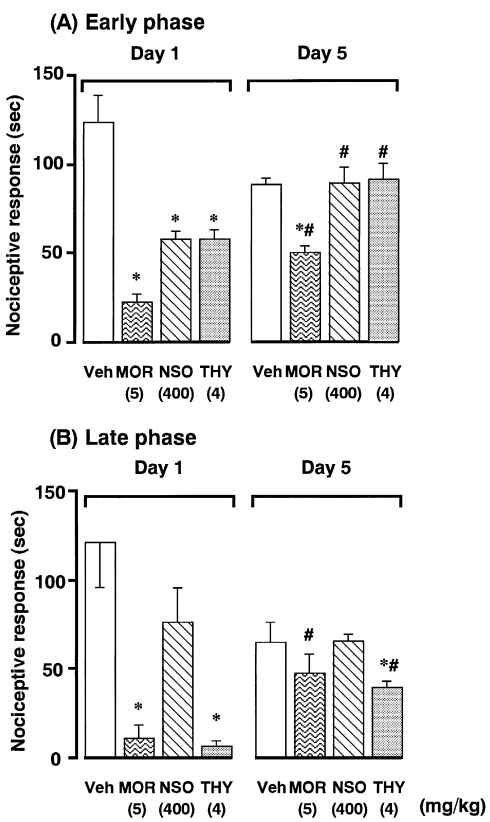

Fig. 7. Development of tolerance to antinociceptive effects of morphine, *N. sativa* seed oil and thymoquinone in the formalin test. Mice received either a single injection or repeated injections, once daily for 5 days and were tested on day 1 and day 5. After the last injection of the test agents, 2% formalin solution was injected into the plantar area of the hindlimb, and the nociceptive responses in the early (A) and late phases (B) were recorded as in Fig. 3. Morphine (MOR: 5 mg/kg, s.c.), thymoquinone (THY: 4 mg/kg, i.p.) and *N. sativa* oil (NSO: 400 mg/kg, p.o.) were administered 30, 30 and 60 min, respectively, before formalin injection. Each column represents the mean ± S.E.M. for 10–12 mice. $^{*}P < 0.05$ compared to the respective vehicle control. $^{\#}P < 0.05$ compared to the respective day 1 group.

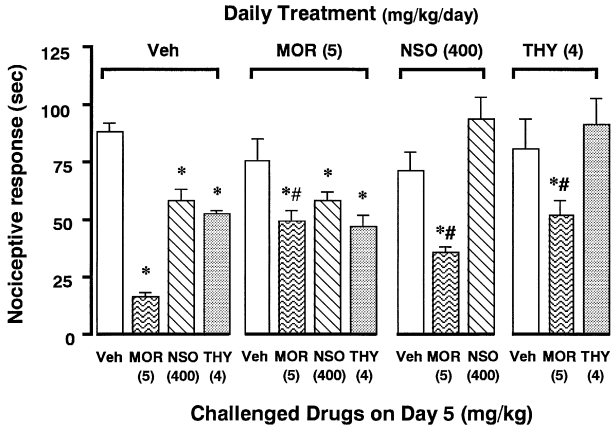

Fig. 8. Asymmetric cross-tolerance between *N. sativa* seed oil-, thymoquinone- and morphine-induced antinociception in the early phase response in the formalin test. Mice received daily vehicle (Veh), morphine (MOR, 5 mg/kg, s.c.), *N. sativa* oil (400 mg/kg, p.o.) or thymoquinone (THY, 4 mg/kg, i.p.) for 4 days before the start of the experiments. On day 5, the animals were challenged with either Veh, morphine (5 mg/kg, s.c.), *N. sativa* oil (400 mg/kg, p.o.), or thymoquinone (4 mg/kg, i.p.) before formalin injection. In the experiments, 2% formalin was injected into the planter area of the hindlimb, and the nociceptive responses in the early phase were recorded for 10 min. Each data column represents the mean ± S.E.M. for 10–12 mice. $^{*}P < 0.05$ compared to the respective vehicle control. $^{\#}P < 0.05$ compared to a single injection of test drugs (5 mg/kg, i.p.).

mice were challenged with morphine, a significant attenuation of morphine-induced antinociception was observed.

4. Discussion

The present study demonstrated that the oral administration of *N. sativa* oil extracted from Egyptian *N. sativa* seeds produces a suppressive effect on nociceptive responses caused by thermal, mechanical and chemical nociceptive stimuli in mice, and that the antinociceptive effect of *N. sativa* oil is partly attributable to its component, thymoquinone. The present results also revealed that at least the supraspinal opioid systems are involved in the antinociceptive effect of thymoquinone.

It has been demonstrated that nociceptive responses caused by acetic acid and formalin can be suppressed, not only by narcotic drugs but also by drugs with anti-inflammatory activity (Hunskaar and Hole, 1987; Shibata et al., 1989; Reanmongkol et al., 1994; Nozaki-Taguchi and Yamamoto, 1998). In this study, p.o. administered *N. sativa* oil significantly inhibited the behavioral changes caused by acute nociceptive stimuli (hot plate, tail-pinch and the early phase of the formalin test) and the prolonged and inflammatory nociception (acetic acid writhing) without affecting spontaneous motor activity in mice. These results suggest that *N. sativa* oil-induced antinociception is due to an inhibitory effect of this oil on the nociceptive systems and/or inflammatory mediators rather than to a sedative effect. The finding that the effect of *N. sativa* oil in the formalin test was reversed by the opioid receptor antagonist, naloxone, provides further support for this idea, and suggests the involvement of opioid systems in *N. sativa* oil-induced antinociception in mice.

Since it is generally considered that nociceptive responses in the formalin test are clinically relevant to forms of continuous pain (Calcagnetti et al., 1988), we used this test in further experiments with thymoquinone. It is interesting to note that systemic and i.c.v. injections of thymoquinone showed a dose-dependent antinociceptive effect on the early and late phase of formalin responses, as do centrally acting antinociceptive drugs (Hunskaar and Hole, 1987; Shibata et al., 1989) and that the effect of i.p. thymoquinone on the early phase response was s.c. and i.c.v. naloxone-reversible. These findings indicate that thymoquinone itself can exert antinociceptive activity via a central mechanism, and suggest that thymoquinone plays an important role in the antinociceptive effect of *N. sativa* oil. Moreover, the antagonistic interaction between thymoquinone and naloxone in the early phase formalin response makes it conceivable that the supraspinal opioid receptors are implicated in thymoquinone-induced antinociception in the early phase response to formalin.

The present pharmacological analysis using selective opioid receptor antagonists revealed that the block of supraspinal μ_1- and κ-but not δ-opioid receptor subtypes attenuated thymoquinone antinociception in the early phase of the formalin test, while none of these receptor subtypes was implicated in the antinociceptive effect of thymoquinone in the late phase response. There is evidence that the early phase of the response to formalin can be inhibited by stimulation of central opioid receptors, while inhibition of the late phase response involves both peripheral and central opioid receptors (Oluyomi et al., 1992). Moreover, μ- and κ-, but not δ-opioid receptors, appear to modulate tonic pain perception at both spinal and supraspinal loci in mice (Calcagnetti et al., 1988; Murray and Cowan, 1991; Fujibayashi et al., 1996), although there have been conflicting reports (Abbott, 1990; Malmberg and Yaksh, 1993; Noble et al., 1995). The reversal of thymoquinone antinociception in the early phase by i.c.v. injections of μ_1- and κ-opioid receptor antagonists suggests that the effect of thymoquinone is at least partly mediated by stimulation of supraspinal μ_1- and κ-opioid receptors.

It remains unclear if thymoquinone antinociception in the formalin test is due to its direct interaction with opioid receptors, particularly μ_1- and κ-opioid receptors, since no information is available regarding the in vitro opioid receptor binding of thymoquinone. However, the difference in naloxone sensitivity of thymoquinone antinociception between the early and late phases raises the possibility that the antinociceptive action of thymoquinone in the formalin test is mediated by mechanism(s) other than direct stimulation of μ- and κ-opioid receptor subtypes located in the

central nervous system. Although i.c.v. thymoquinone attenuated the late phase response to formalin, we cannot exclude the possibility that i.p. thymoquinone-induced antinociception in the late phase is partly mediated by peripheral mechanisms. A previous study (Houghton et al., 1995) revealed that thymoquinone as well as *N. sativa* oil inhibited the generation of eicosanoids such as thromboxane B_2 and leukotriene B_4. As drugs, which suppress these inflammatory mediators, attenuate the late phase nociceptive responses in the formalin test (Hunskaar et al., 1986; Reanmongkol et al., 1994; Taniguchi et al., 1994; Matsuda et al., 1998), one might infer that the anti-inflammatory effect of thymoquinone is implicated in its antinociceptive effect in the late phase. Such a possibility is presently under investigation in this laboratory.

In the present study, morphine tolerance in the formalin test was observed after once-daily injections of morphine for 4 days. These results are consistent with the previous findings that tolerance to morphine antinociception in the formalin test develops in 2 days of twice-daily injections (Connell et al., 1994; Detweiler et al., 1995), although there is a conflicting report (Abbot et al., 1981). Moreover, the present study revealed that, when administered repeatedly, *N. sativa* oil and thymoquinone also had tolerance develop to their antinociceptive action in the early phase and both phases of the formalin test, respectively. The exact mechanism underlying the development of tolerance to *N. sativa* oil- and thymoquinone-induced antinociception in the formalin test is unclear, but one possibility is that *N. sativa* oil and thymoquinone produce antinociception in the formalin test by releasing endogenous opioid peptides in the central nervous system, and cause antinociceptive tolerance following repeated administration. In this study, although the antinociceptive effect of morphine was reduced in thymoquinone- and *N. sativa* oil-pretreated mice, no cross-tolerance was observed for the antinociceptive effects of *N. sativa* oil and thymoquinone in morphine-tolerant mice. This asymmetric tolerance between *N. sativa* oil- or thymoquinone-induced antinociception and morphine-induced antinociception seems to support the possibility that the antinociceptive effect of *N. sativa* oil and thymoquinone in the formalin test is not due to direct stimulation of the opioid receptors. Nevertheless, further experiments are needed to clarify the mechanism underlying both the antinociceptive action of *N. sativa* oil and thymoquinone, and the asymmetric cross-tolerance between *N. sativa* oil and thymoquinone and morphine in the formalin test.

In conclusion, the present study provided evidence for the first time that *N. sativa* oil and thymoquinone produce antinociceptive effects and that the supraspinal opioid systems, particularly μ_1- and κ-opioid receptor subtypes are involved in the antinociceptive action of *N. sativa* oil and thymoquinone in the early phase of formalin-induced nociceptive responses in mice.

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