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Role of L-arginine/NO/cGMP/K_{ATP} channel signaling pathway in the central and peripheral antinociceptive effect of thymoquinone in rats

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ABSTRACT

Objective(s): Growing evidence demonstrates that L-arginine/NO/cGMP/K_{ATP} channel pathway has a modulatory role in pain perception. Previous studies have shown that thymoquinone exerts antinociceptive effects; however, the mechanisms underlying antinociception induced by thymoquinone have not been fully clarified. The aim of the present study was to evaluate the role of L-arginine/NO/cGMP/K_{ATP} channel pathway in the central and peripheral antinociceptive effect of thymoquinone in rats.

Materials and Methods: Rats were pretreated intraplantarly (IPL) or intracerebroventricularly (ICV) with L-arginine (the NO precursor), l-NAME (an NO synthase inhibitor), SNAP (an NO donor), methylene blue (a guanylyl cyclase inhibitor), glibenclamide (the blocker of K_{ATP} channel), and tetraethylammonium (TEA, a K_v channel blocker) before the injection of thymoquinone.

Results: Local ipsilateral (20 and 40 µg, IPL) but not contralateral and ICV (4 and 8 µg) administration of thymoquinone caused a dose-dependent and significant antinociception in both early and late phases of the formalin test. Pretreatment of rats with L-arginine (100 µg, IPL or ICV) and SNAP (200 µg, IPL or ICV) increased while l-NAME (100 µg, IPL or 1 µg, ICV) and methylene blue (400 µg, IPL or ICV) decreased the antinociceptive effects of thymoquinone in the formalin test. The administration of TEA (IPL or ICV) did not modify but glibenclamide (50 µg, IPL or ICV) significantly abolished the peripheral and central antinociceptive effects of thymoquinone in both phases of the formalin test.

Conclusion: The results of the present study indicate that L-arginine/NO/cGMP/K_{ATP} channel pathway participates in the central and peripheral antinociceptive effect of thymoquinone.

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Introduction

Thymoquinone (2-isopropyl-5-methyl-benzo quinone) is the predominant bioactive constituent found in the medicinal plant, *Nigella sativa*. Structurally, thymoquinone is a monocyclic monoterpene that is extracted from the essential oils of *N. sativa* seeds (1). Monoterpenes, as a substructure of terpenes, are a large and important class of naturally occurring organic compounds, which possess highly distinctive aromas and flavors. More than 90% of the essential oils of medicinal herbs consist of monoterpenes (2). Among monoterpenes, thymoquinone is a unique molecule with several pharmacological effects such as anticonvulsant (3, 4), muscle relaxant (5–7), and neuroprotective (8–11). However, the antinociceptive effect of thymoquinone has not been fully studied.

To date, only two studies have been conducted to evaluate the antinociceptive effect of thymoquinone. Abdel-Fattah *et al.* examined the antinociceptive effects of thymoquinone in an animal model of nociception. Their findings showed that a central mechanism through opioid receptors plays a role in the antinociceptive effect of thymoquinone (12). In addition, de Sousa *et al.* evaluated the antinociceptive effects of thymoquinone and its para-benzoquinones analogs using the formalin test in mice. They showed that thymoquinone decreased nociceptive responses in both phases of the formalin test (13). However, underlying mechanisms through which thymoquinone and its analogs alleviate nociceptive pain were not determined.

Several studies have demonstrated that the signaling pathway of nitric oxide (NO), 3', 5'-cyclic guanosine monophosphate (cGMP) and potassium (K^+) channels, modulate pain perception and participate in the antinociceptive activity of analgesic drugs. In this regard, central and peripheral K^+ channels play a crucial role in the nociceptive processes (14–16). It has been shown that the activation of ATP-sensitive K^+ channels (K_{ATP}) by K_{ATP} channel openers produces antinociceptive effects and potentiates the antinociception induced by analgesic drugs (17–19). In contrast, K_{ATP} channel blockers antagonize the antinociception induced by pain-relieving medications (15, 19-21). On the other hand, pretreatment with L-arginine, a substrate for nitric-oxide synthase, potentiates while the inhibition of the enzymes NO synthase (NOS) and soluble guanylyl

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cyclase antagonizes the antinociception induced by analgesic drugs (15, 21-23). Based on such evidence, it has been hypothesized that the L-arginine/NO/cGMP pathway is of importance in the transduction of pain signaling at central and peripheral levels of nociceptive signaling pathways (24).

Basically, there is a close relationship between K^+ channels and the NO/cGMP pathway, which contributes to antinociceptive mechanisms. K^+ channels can be opened following the activation of the NO/cGMP pathway in the central and peripheral nervous system (14, 24). There is evidence indicating that the NO/cGMP/K_{ATP} channel signaling pathway has a crucial role in the antinociceptive effects of two main classes of analgesic drugs including opioids and NSAIDs (non-steroidal anti-inflammatory drugs) (21–25).

The present study was carried out to evaluate the central and peripheral antinociceptive effects of thymoquinone and to investigate, by means of pharmacological approaches, the role of the NO/cGMP/K_{ATP} channel pathway in its analgesic effects. Thereby, the current study provides further insights into the mechanism of antinociception induced by thymoquinone. Having more information about the mode of action of an analgesic agent would certainly lead us to its better clinical application.

Materials and Methods

Animals

In this study, male Wistar rats weighing 250–270 g were used. We kept the rats in standard plastic cages on a 12 hr light/12 hr dark cycle at 23±2 ˚C. The animals had easy and free access to a water bottle and a food pellets container at all times. The animals were randomly divided into groups of 7 each and were used only once throughout the experiments. They were acclimatized and habituated to the laboratory environment for at least 1 hr before testing. All stages of experiments were carried out under the precise supervision of the Ethical Committee in the School of Medicine, Shahid Beheshti University of Medical Sciences.

Drugs and chemicals

In this study, thymoquinone, glibenclamide (the K_{ATP} channel blocker), tetraethylammonium (TEA, a K_v channel blocker), l-NAME (the NOS inhibitor), L-arginine (the NO precursor), SNAP (S-nitroso-N-acetylpenicillamine, the NO donor), and methylene blue (the inhibitor of soluble guanylyl cyclase) were used. All compounds were purchased from the Sigma-Aldrich Co. (St Louis, MO, USA) except for glibenclamide, which was procured from the Poursina Pharmaceutical Co. (Tehran, Iran). Drugs were dissolved in normal saline (NaCl 0.9%). Thymoquinone and glibenclamide were suspended in tween 80 (0.5%, v/v). All drug solutions were prepared freshly and administered intraplantarly (IPL) or intracerebroventricularly (ICV) in a volume of 50 µl and 5 µl, respectively. Control rats received normal saline only or with tween 80 as vehicle. Dosage and the schedules of drug administration were selected based on previous studies (25–29).

Experimental procedures for cannulation and ICV drug injection

For ICV drug injection, guide cannulas were stereotaxically implanted in the right ventricle of the rat brains as described previously (3). Briefly, the rats were anesthetized with ketamine (60 mg/kg, IP) and xylazine (6 mg/kg, IP) and then mounted on a stereotaxic frame (Stoelting Stereotaxic Apparatus, USA). Subsequently, a 21-gauge, 9-mm stainless steel guide cannula was implanted in the right lateral ventricle of the brain (AP: -0.9 mm from bregma; ML: +1.6 mm from midline; DV: 2.4 mm from the skull surface) (30). The cannulas were fastened to the skull surface using dental acrylic cement and small stainless steel screws.

The rats were allowed 7 days to recover after surgery. On the day of the experiments, the ICV injections of thymoquinone and controls were performed using a Hamilton syringe (10 µl) connected to a 26-gauge stainless steel injector by a polyethylene tube. We kept the injector in place for another 1 min after the end of ICV injection to ensure the completion of drug infusion.

Formalin test

The formalin test was used to evaluate the antinociceptive effect of thymoquinone and the underlying mechanisms. Rats were first placed in an open observation chamber (Plexiglass; 30×30×40 cm) for 5–10 min to acclimatize to their surroundings. Then, 50 µl of formalin (2.5% in normal saline) was injected IPL into the right hind paw of rats with a fine needle (30-gauge). The animals were immediately observed for nociceptive response following the formalin injection. The licking time of right hind paw was recorded as nociceptive response. A mirror was placed under the cage at an angle of 45° to enable full view of the formalin-injected hind paw. The paw licking time was recorded at intervals of 5 min during 30 min (31).

Antinociceptive effect of thymoquinone

To evaluate the peripheral antinociceptive effect of thymoquinone, rats received unilateral IPL injection of thymoquinone (10, 20, and 40 µg/paw), vehicle, or morphine (10 µg/paw) 20 min before the injection of formalin into the right hind paw (ipsilateral). To clarify whether the antinociceptive effect of thymoquinone acted locally, in one more group of rats, thymoquinone (20 µg/paw) was administered to the left hind paw (contralateral) 20 min before the injection of formalin to the right paw. Moreover, to evaluate the central antinociceptive effect of thymoquinone, rats received an ICV injection of thymoquinone (2, 4, and 8 µg/rat) or appropriate vehicle 20 min before the formalin test. Morphine (4 µg/rat, ICV) was used as a reference analgesic drug.

Evaluation of the role of the signaling pathway of NO/cGMP/K_{ATP} channel in antinociception induced by thymoquinone

Role of L-arginine/NO pathway

To clarify the involvement of L-arginine/NO pathway in the peripheral antinociception induced by local administration of thymoquinone, first L-arginine (100 µg/paw), l-NAME (100 µg/paw), or SNAP (200 µg/paw) were injected IPL into the right hind paw. After 15 min, thymoquinone (20 µg/paw) was administered IPL into the same hind paw. The nociceptive responses were evaluated 20 min after the injection of thymoquinone

using a formalin test. To determine the role of L-arginine/NO pathway in the central antinociceptive effect of thymoquinone, rats were pretreated by ICV injection of L-arginine (100 µg/rat), l-NAME (1 µg/rat), or SNAP (200 µg/rat) 15 min before thymoquinone administration (8 µg/rat, ICV). The formalin test was carried out 20 min after thymoquinone administration.

Role of cGMP

In order to assess the role of cGMP in the peripheral and central antinociceptive effect of thymoquinone, methylene blue as an inhibitor of soluble guanylyl cyclase was injected IPL (400 µg/paw) or ICV (400 µg/rat) 15 min before the administration of thymoquinone (20 µg, IPL and 8 µg, ICV, respectively). Twenty min after the peripheral or central injection of thymoquinone, formalin was injected in the right hind paw of rats and the nociceptive response was evaluated. Since nitric oxide activates guanylyl cyclase in the nervous system, which in turn increases cGMP (32), we further examined whether the antinociceptive effect of thymoquinone was associated with an increase in NO followed by elevated cGMP levels. In this phase of the experiments, the rats received first an ICV dose of methylene blue (400 µg/rat) 60 min prior to the injection of SNAP (200 µg/rat, ICV). Then, thymoquinone (8 µg/rat, ICV) was injected 15 min after the administration of SNAP and finally, the formalin assay was performed 20 min following thymoquinone administration. Furthermore, to assess the relationship between NO and cGMP in the peripheral antinociceptive effect of thymoquinone, methylene blue (400 µg/paw) was administered IPL 1 hr before the injection of SNAP (200 µg/paw). Then, thymoquinone (20 µg/paw) was injected into the same right paw 15 min after the administration of SNAP. Finally, the formalin test was carried out 20 min after the injection of thymoquinone.

Role of potassium channels

To determine whether the antinociceptive effect of thymoquinone was mediated through the participation of K^+ channel activation, glibenclamide (the inhibitor of K_{ATP} channel) and TEA (the inhibitor of the voltage-dependent K^+ channel) were used. Rats were pretreated with IPL injection of glibenclamide (50 µg/paw) or TEA (100 µg/paw) 15 min before the IPL administration of thymoquinone (20 µg/paw) into the same paw. The nociceptive responses were evaluated 20 min after the injection of thymoquinone using the formalin test. To ascertain whether K^+ channels participated in the central antinociceptive effect of thymoquinone, rats were first treated with an ICV injection of glibenclamide (50 µg/rat) or TEA (0.5 µg/rat). After 15 min, thymoquinone (8 µg/rat) was injected ICV and the nociceptive behavior was evaluated through the formalin test.

Evaluation of locomotor activity

The effect of thymoquinone on the locomotor activity of the animals was assessed using the open-field test as described earlier (33). We used a white wooden box with a floor of 100 × 100 cm and walls of 30 cm high as the open-field. The floor of the box was divided equally into 25 squares of 20 × 20 cm using red lines. Twenty min after the administration of thymoquinone (10, 20, and 40 µg, IPL or 2, 4, and 8 µg, ICV) or vehicle, the animals were placed in the central square of the open-field floor and their locomotor activity was monitored for 10 min. The total number of squares crossed by each rat (total locomotion) was recorded.

Statistical analysis

The data obtained from the experiments were expressed as mean ± SEM for 7 rats per group. To compare the mean of the nociceptive response of each group with all other groups, we performed the ANOVA (analysis of variance), which was followed by the Tukey's test. The differences between the means of the nociceptive responses of the groups were considered significant when $P<0.05$.

Results

Antinociceptive effects of thymoquinone in the formalin test

The IPL injection of formalin in rats induced a typical nociception characterized by a biphasic time-course. The early phase (neurogenic phase) began after the IPL injection of formalin and persisted for 5 min. The late (inflammatory) phase started at about 15 min following the IPL injection of formalin and gradually decreased within 15 min. A short quiescent interval (about 10 min) between the two phases was observed in rats characterized by at least a nociceptive response. The time-course of pain response in rats that received thymoquinone (2–8 µg, ICV or 10–40 µg, IPL) is shown in Figure 1.

The local ipsilateral administration of thymoquinone (20 and 40 µg/paw) caused a significant reduction in the paw licking time in both the early and late phases of the formalin assay (Figures 2A, B). The IPL administration of thymoquinone also produced an antinociceptive effect in the early phase of the formalin test in a dose-dependent manner (Figure 2A). As shown in Figures 2A and B, contralateral administration of thymoquinone (40 µg/paw) did not produce any antinociceptive effect. In addition, the ICV administration of thymoquinone (4 and 8 µg/rat) caused a dose-dependent and significant decrease in nociceptive response in both phases of the formalin test (Figures 2C, D).

Thymoquinone in low doses (2 µg, ICV or 10 µg/paw) did not elicit any antinociceptive effect. Based on these results, the effective doses of thymoquinone (20 µg/paw and 8 µg, ICV) were used to assess the role of the signaling pathway of the NO/cGMP/K_{ATP} channel in the

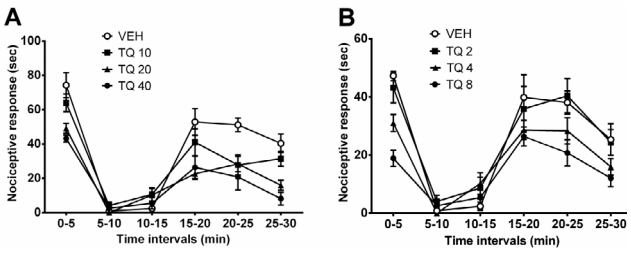

Figure 1. Time-response relationship of antinociception induced by thymoquinone (TQ) in the rat formalin test. TQ was administered intraplantarly (A) or intracerebroventricularly (B) 20 min before the intraplantar injection of formalin. Data are expressed as the means ± SEM for 7 rats. VEH: vehicle (normal saline + tween 80)

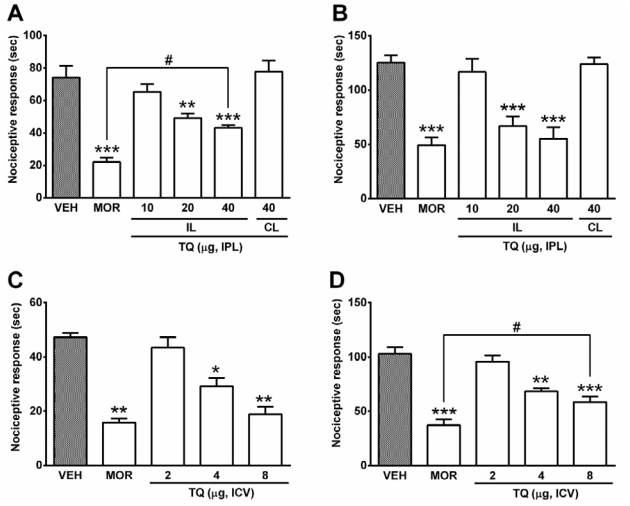

Figure 2. Dose-response relationship for antinociception induced by thymoquinone (TQ) in the formalin test. A significant reduction in the nociceptive response was observed 20 min after the IPL (A, B) or ICV (C, D) administration of TQ in both first (A, C) and second (B, D) phases of the formalin assay. Morphine (10 µg, IPL or 4 µg, ICV) was used as the standard analgesic drug. Data are expressed as the means ± SEM for 7 animals. VEH: vehicle (normal saline + tween 80), IL: ipsilateral, CL: contralateral, IPL: intraplantar, ICV: intracerebroventricular. * P<0.05, ** P<0.01, *** P<0.001 compared to VEH, # P<0.05 compared to morphine

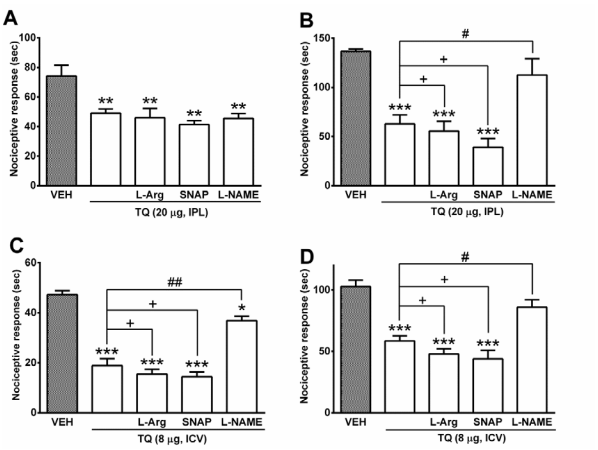

Figure 3. Role of nitric oxide (NO) on peripheral (A, B) and central (C, D) antinociceptive effect of thymoquinone (TQ) in the first (A, C) and second (B, D) phases of formalin test in rats. L-Arg (100 µg, IPL or ICV), L-NAME (100 µg, IPL or 1 µg, ICV), and SNAP (200 µg, IPL or ICV) were administered 15 min before the injection of TQ. The formalin test was carried out 20 min following the administration of TQ. Data are expressed as the means ± SEM for 7 animals. VEH: vehicle (normal saline + tween 80), L-Arg: L-arginine, SNAP: S-nitroso-N-acetylpenicillamine, L-NAME: N(G)-Nitro-L-arginine methyl ester, IPL: intraplantarly, ICV: intracerebroventricularly. ** P<0.01, *** P<0.001 compared to VEH, # P<0.05, ## P<0.01, + P>0.05

antinociceptive effects of thymoquinone.

Morphine, as the reference drug, significantly inhibited the pain response in both phases of formalin assay (Figure 2). There was also a significant difference between the antinociceptive effects of morphine and thymoquinone. The results showed that IPL administration of morphine (10 µg/paw) was significantly more effective than thymoquinone in the early phase (P<0.05; Figure 2A). However, the peripheral antinociceptive effect of thymoquinone was as effective as morphine in the late phase of the formalin test because no significant difference was found between rats receiving thymoquinone (20, 40 µg/paw) and those treated with morphine (10 µg/paw) (Figure 2B). In addition, a significant difference was seen between antinociception induced by ICV injection of morphine (4 µg/rat) and thymoquinone (8 µg/rat) in the late phase (P<0.05; Figure 2D), but not in the early phase of formalin test (Figure 2C). The results showed that the antinociception induced by the ICV administration of thymoquinone (8 µg/rat) only in the first 5 min of the formalin test is comparable to that of morphine (4 µg/rat, ICV), because no significant difference was found between them (P = 0.929; Figure 2C). No adverse effects were observed following the administration of thymoquinone and controls in rats.

Involvement of L-arginine/NO pathway

Intraplantar injection of l-NAME (100 µg/paw), the NOS inhibitor, significantly diminished the antinociceptive effect of thymoquinone (20 µg/paw) in the second phase of the formalin test (P<0.05; Figures 3A, B). In addition, the ICV administration of l-NAME (1 µg/rat), significantly antagonized the antinociceptive effects of thymoquinone (8 µg/rat, ICV) in both phases of the formalin assay (P<0.01; Figure 3C and P<0.05; Figure 3D, respectively). On the other hand, local injection of 100 µg/paw L-arginine (the NO precursor) and 200 µg/paw SNAP (the NO donor) into the right hind paw increased the antinociceptive effects of thymoquinone in both phases of the formalin test, but these changes were not statistically significant (Figures 3A, B). The results also showed that following the ICV administration of L-arginine (100 µg/rat), the antinociceptive effects of thymoquinone (8 µg/rat, ICV) in the first and second phases of the formalin test increased by 18.0% and 18.2%, respectively, but the difference between the mean of the nociceptive response of the two groups was not statistically significant (Figures 3C, D). Pretreatment of rats with SNAP (200 µg/rat, ICV) caused a non-significant increase in the central antinociceptive effect of thymoquinone in the first and second phases of the formalin test by 23.9% and 24.8%, respectively (Figures 3C, D).

The administration of L-arginine (100 µg, IPL or ICV), l-NAME (100 µg, IPL; 1 µg, ICV), and SNAP (200 µg, IPL or ICV) did not affect the nociceptive response in the formalin test (Figure 4). The administration of low doses of L-arginine (25 and 50 µg ICV or IPL) and l-NAME (0.25 and 0.5 µg, ICV or 25 and 50 µg, IPL) neither affected the nociceptive response nor modified the antinociceptive effect of thymoquinone (data not shown). Low doses of SNAP (50 and 100 µg, ICV or IPL) also did not have any effect on the nociceptive response in the formalin test and could not modify the antinociception induced by thymoquinone (data not shown).

Involvement of cGMP

The local administration of methylene blue (400 µg, IPL) significantly decreased the peripheral antinociceptive effect of thymoquinone (20 µg/paw) in the formalin test (P<0.05; Figures 5A, B). In addition, the central antinociceptive effect of thymoquinone (8 µg/

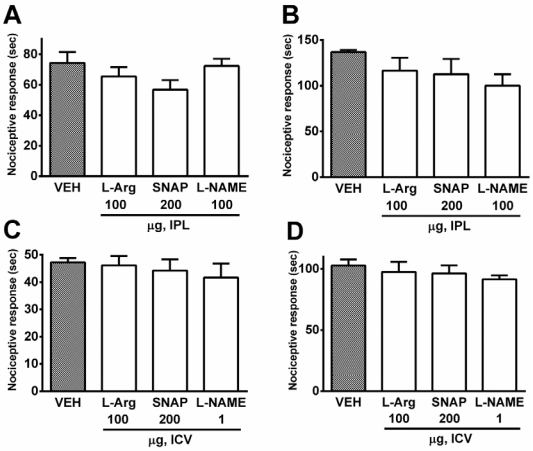

Figure 4. The effect of intraplantar (IPL) or intracerebroventricular (ICV) administration of l-arginine (L-Arg), S-nitroso-N-acetylpenicillamine (SNAP), and N(G)-Nitro-L-arginine methyl ester (L-NAME) on formalin-induced nociception in rats. Data are expressed as the means ± SEM for 7 rats. VEH: vehicle (normal saline + tween 80)

rat, ICV) was antagonized by methylene blue (400 µg/rat, ICV) in the first 5 min of the formalin test (P<0.05; Figure 5C). Methylene blue (400 µg/rat, ICV) also resulted in the diminution of the antinociceptive effect of thymoquinone in the second phase of the formalin assay (Figure 5D); however, this effect was not statistically significant (P = 0.333). Lower doses of methylene blue (100 and 200 µg), when administered ICV or IPL, could not modify antinociception induced by thymoquinone. None of the doses of methylene blue (100–400 µg), when used alone, could affect nociceptive response in the formalin test (data not shown). Administration of SNAP (either ICV or IPL) in methylene blue-treated rats could not restore the antinociceptive effect of thymoquinone in any of the phases of the formalin test (Figure 5).

Involvement of potassium channel

The administration of the TEA (the blocker of voltage-dependent potassium channels), either ICV or IPL, did not modify the nociceptive responses of thymoquinone-treated rats (Figure 6). In contrast, the IPL administration

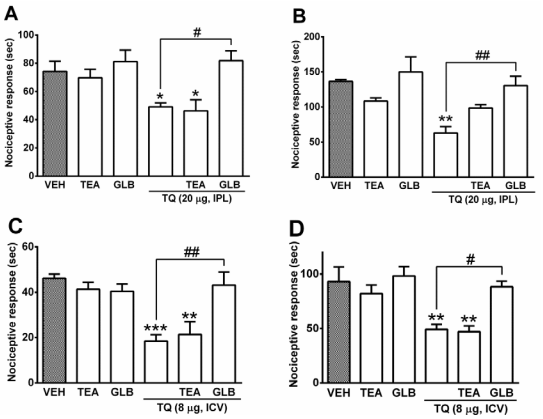

Figure 6. The effect of glibenclamide (GLB) tetraethylammonium (TEA) on peripheral (A, B) and central (C, D) antinociception induced by thymoquinone (TQ) in the first (A, C) and second (B, D) phases of formalin assay in rats. GLB (50 µg, IPL or ICV) and TEA (100 µg, IPL or 0.5 µg, ICV) were administered 15 min before TQ. The formalin test was carried out 20 min after the injection of TQ. Data are expressed as the means ± SEM for 7 animals. VEH: vehicle (normal saline + tween 80), IPL: intraplantarly, ICV: intracerebroventricularly, * P<0.05, ** P<0.01, *** P<0.001 compared to VEH, # P<0.05, ## P<0.01

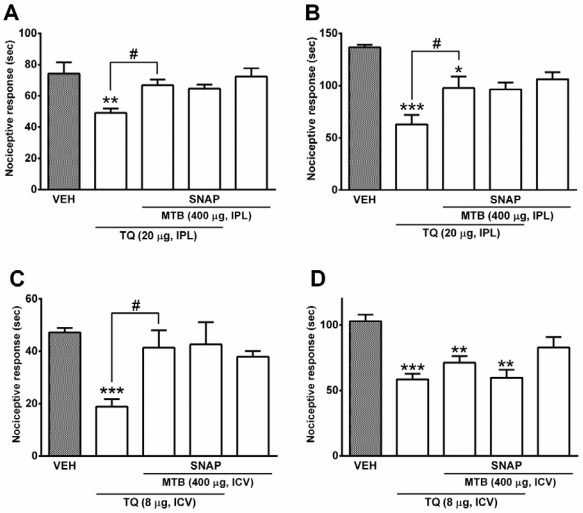

Figure 5. The effect of methylene blue (MTB) a guanylyl cyclase inhibitor on peripheral (A, B) and central (C, D) antinociceptive effect of thymoquinone (TQ) in the first (A, C) and second (B, D) phases of formalin tests in rats. MTB was administered 15 min before TQ. In the SNAP-treated group, rats were first treated with MTB and then received SNAP (200 µg, IPL or ICV) after 60 min in the SNAP-treated group, TQ was given 15 min after SNAP. The formalin test was performed 20 min after the TQ administration. Data are expressed as the means ± SEM for 7 rats. VEH: vehicle (normal saline + tween 80), SNAP: S-nitroso-N-acetylpenicillamine, IPL: intraplantarly, ICV: intracerebroventricularly, * P<0.05, ** P<0.01, *** P<0.001 compared to VEH, # P<0.05

of glibenclamide (50 µg/paw) significantly abolished the peripheral antinociceptive effect of thymoquinone in both the first and second phases of the formalin assay (P<0.05; Figure 6A and P<0.01; Figure 6B, respectively). Similar results were found with the ICV injection of glibenclamide. As shown in Figure 6, glibenclamide (50 µg/rat, ICV) significantly reversed the antinociception induced by ICV administration of thymoquinone (8 µg) in the early (P<0.01) and late (P<0.05) phases of the formalin test (Figures 6C, D). Intraplantar or ICV administration of glibenclamide (50 µg) alone did not produce any antinociceptive effect in the formalin test (Figure 6). Lower doses of glibenclamide (12.5 and 25 µg, either ICV or IPL) could not modify the antinociceptive effect of thymoquinone (data not shown).

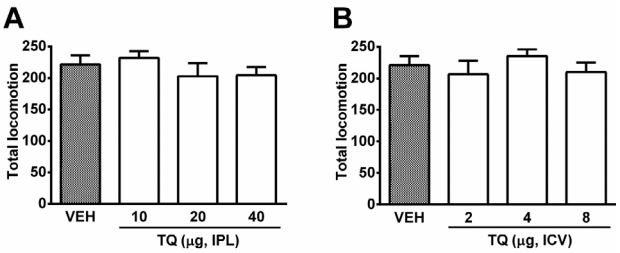

Figure 7. The effect of different doses of thymoquinone on locomotor activity of rats. Twenty min after the intraplantar (IPL) or intracerebroventricular (ICV) administration of thymoquinone or vehicle (VEH), the rats were placed in the central square of the open-field and total locomotion was recorded during 10 min. Data are expressed as the means ± SEM for 7 animals. VEH: normal saline + tween 80

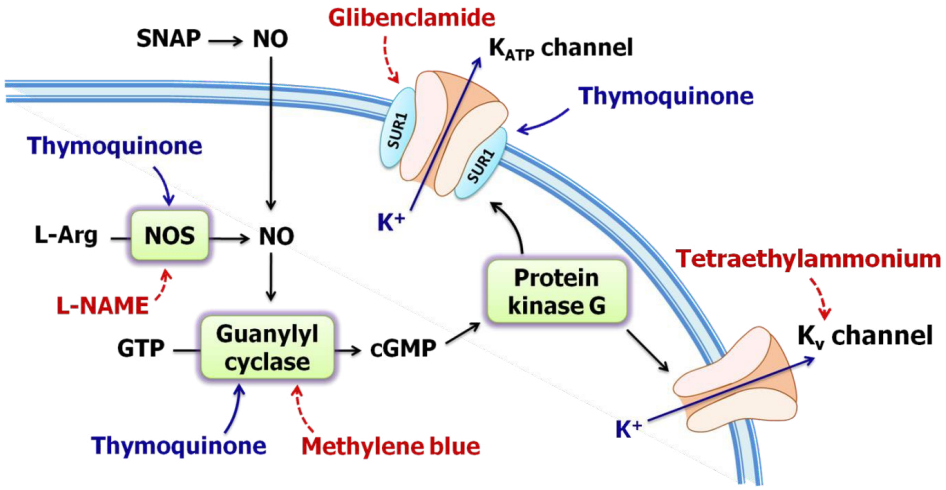

Figure 8. Possible sites of action of thymoquinone in the NO/cGMP/K_{ATP} channel pathway. Nitric oxide (NO) is either released exogenously by SNAP (S-nitroso-N-acetylpenicillamine, the NO donor) or synthesized endogenously from its major precursor L-arginine (L-Arg) by the enzyme nitric oxide synthase (NOS). NO can activate the enzyme soluble guanylyl cyclase resulting in an increase in the cyclic guanosine monophosphate (cGMP) synthesis. Cyclic GMP, as a second messenger, modulates various intracellular targets such as PKG (cGMP-dependent protein kinase) and cGMP-regulated K_{ATP} and K_v channels, which contribute to the synaptic transmission of pain impulses in the central and peripheral nervous systems. Dashed arrows indicate an inhibitory effect. SUR1: sulfonylurea receptor-1

Effect of thymoquinone on locomotor activity

In the open-field test, different doses of thymoquinone (2–8 µg, ICV or 10–40 µg, IPL) had no significant effect on the total locomotion of rats (Figures 7A, B).

Discussion

The present study showed that the central or peripheral administration of thymoquinone could alleviate formalin-induced pain in rats through the modification of NO/cGMP/K_{ATP} channel signaling pathway.

Our results indicate that the ICV administration of thymoquinone in rats decreased the pain intensity in both neurogenic and inflammatory phases of the formalin test in a dose-dependent manner so that an increase in the dose of thymoquinone gradually decreased the pain response in rats. We also compared the antinociceptive effect of thymoquinone with morphine. Although morphine exerts its analgesic effect by stimulating the opioid receptors, there is evidence indicating the involvement of the K_{ATP} channels in the analgesic effect of morphine (20, 34). Hence, in this study morphine was considered the standard drug (positive control). Our results also indicated that a high dose of thymoquinone (8 µg/rat, ICV) exerted antinociception as effective as morphine in the first 5 min of the formalin test. The ICV effective dose of thymoquinone in this study was 6 to 12 times higher than the amount reported for anticonvulsant efficacy in previous studies (3). These observations reinforce the notion that thymoquinone exerts dose-dependent neuroprotective effects.

In addition, the present study has shown for the first time that the local administration of thymoquinone in the plantar surface of the hind paw produces a significant antinociceptive effect in the formalin assay. The peripheral antinociceptive effect of thymoquinone in the early phase of formalin test was dose-dependent. The peripheral antinociceptive effect of thymoquinone appeared not to be mediated through the central mechanisms because contralateral administration of thymoquinone could not relieve formalin-induced pain. This finding indicates that thymoquinone acts locally as an antinociceptive agent in the formalin test when given subcutaneously into the rats' paw. The antinociception induced by the IPL administration of thymoquinone in the second phase of the formalin test was similar to that of morphine. However, morphine produced more effective antinociception than higher doses of thymoquinone in the first phase of the formalin assay.

The mechanisms underlying antinociception induced by thymoquinone had not been fully clarified previously. Abdel-Fattah *et al.* had previously reported that the opioid system may have a role in the antinociceptive effects of thymoquinone (12). However, other underlying mechanisms, especially the role of the signaling pathway of L-arginine/NO/cGMP/K_{ATP} channel in antinociception induced by thymoquinone, had not been investigated in the past.

Recently, particular attention has been focused on the modulatory role of the L-arginine/NO/cGMP/K_{ATP} channel pathway in nociceptors and pain sensitization in the central and peripheral nervous system. NO appears to have a dual effect on the pain threshold, depending on its concentration, target tissue, and animal model of pain (24, 35). There is evidence that the IPL injection of formalin in mice may locally activate NOS and increase NO levels. This may provoke nociceptive behavior in mice in the inflammatory phase of the formalin test (35). Nevertheless, the precise role of NO in modulation of pain is controversial. It has been shown that L-arginine, the precursor of NO, exerts pro-nociceptive effects at low doses, while at high doses it exhibits antinociceptive effects in formalin- and carrageenin-induced hyperalgesia in rats (35, 36). L-arginine, at doses that do not modify nociceptive response, may enhance the efficacy of various analgesic agents in the formalin test (29, 37). A higher dose of L-arginine (0.2–1 mg, ICV or IPL) is needed to produce antinociceptive effects (36). In contrast, l-NAME, the inhibitor of nitric oxide synthesis, with non-analgesic doses, reverses the

pain-relieving effects of different analgesic drugs (21, 25, 38, 39). Consistent with these reports, our results indicated that the administration of non-analgesic doses of L-arginine enhanced, while l-NAME reversed, the antinociceptive effect of thymoquinone. These findings demonstrate that NO contributed to the peripheral and central mechanism of antinociceptive effects of thymoquinone. Similar results were obtained in a SNAP-treated group. SNAP, an NO donor, partially increased the antinociceptive effect of thymoquinone. This finding reinforces the contribution of NO to thymoquinone-induced analgesia. Previous studies have shown that SNAP and other non-enzymatic donors of nitric oxide such as SIN-1 and sodium nitroprusside potentiate the antinociceptive effects of several analgesic agents (25, 29, 36, 37, 40). There are even pieces of clinical evidence indicating that NO donors can efficiently relieve local pain (41).

Cyclic GMP has a close relationship with NO in the pain-related signaling pathway. When the NO level is increased, the enzyme soluble guanylyl cyclase will be activated, thus resulting in an increase in cGMP synthesis. Cyclic GMP, as a second messenger, modulates various intracellular targets such as PKG (cGMP-dependent protein kinase-I) and cGMP-regulated ion channels which contribute to the biosynthesis of substance P and synaptic transmission of pain impulses in the CNS (24). The participation of cGMP and NO in analgesic effects of different groups of drugs such as tramadol (23), gabapentin (28), indomethacin (42), and diclofenac (21) has been previously demonstrated.

Methylene blue, a well-known inhibitor of guanylyl cyclase, is often used to reveal the contribution of cGMP in pain signaling and clarification of the mechanism of the antinociceptive effect of pain-relieving medications (29, 43, 44). The results of the present study show that the signaling pathway of NO/cGMP participates in the antinociceptive effects of thymoquinone because pretreatment of rats with methylene blue reversed the antinociception induced by ICV or IPL injection of thymoquinone in the formalin test. These results indicate that guanylyl cyclase-mediated biosynthesis of cGMP is needed for the antinociceptive effect of thymoquinone. In addition, the administration of SNAP (the NO donor) following the inhibition of guanylyl cyclase could not restore the antinociceptive effect of thymoquinone. This finding demonstrates that the enzyme guanylyl cyclase is required for the potentiating effect of NO on central and peripheral antinociception induced by thymoquinone. Our finding regarding the interaction of thymoquinone with the NO/cGMP pathway is consistent with the report by Gilhotra and Dhingra (2011). They have recently shown that the anxiolytic effect of thymoquinone is mediated through the modification of NO/cGMP pathways in mice (45).

Several cellular targets including potassium channels have been identified for cGMP (24). There is evidence indicating that the activation of the NO/cGMP pathway promotes the opening of K^+ channels, which result in the hyperpolarization of neuron membranes. In this regard, the involvement of K_{ATP} channels in the antinociceptive effects of two major analgesic drugs including opioids and NSAIDs have been demonstrated (18, 20, 25, 34, 46). There is also evidence indicating that K^+ channel openers exert remarkable analgesic effects in the treatment of chronic pains (47). Because of the importance of potassium channels, especially neuronal K_{ATP} and K_v7 in the processing of pain signaling, it has been suggested that potassium channels could be considered a new drug target for developing novel analgesic drugs (16).

The findings of the present study demonstrate that K_{ATP} channels contribute to the antinociception induced by thymoquinone. Our results showed that the ICV or IPL administration of glibenclamide, which inhibits sulfonylurea receptor-1 (SUR1) in the K_{ATP} channels (48), antagonized the antinociceptive effects of thymoquinone. However, ICV or IPL injection of tetraethylammonium chloride as the K_v channel blocker failed to abolish the antinociceptive effects of thymoquinone. These results are consistent with previous studies reporting the participation of K_{ATP} channels in the pharmacological effects of thymoquinone. Suddek (2010) showed that the relaxation of isolated arterial rings by thymoquinone is mediated through the activation of the K_{ATP} channels (49).

Our observations from the open-field test showed that thymoquinone did not have any effect on the total locomotion in the rats. This indicates that thymoquinone did not impair the locomotor activities of the rats and, thereby, did not have any influence on pain responses of the animals in the formalin test.

Conclusion

The results of the present study demonstrate that central or peripheral administration of thymoquinone attenuates formalin-induced pain through modification of the NO/cGMP/K_{ATP} channel pathway (Figure 8).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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