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Original Article

The Possible Anti-nociceptive Effect of Troxerutin and Neural Interactions Using Formalin and Writhing Tests in Mice

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Abstract

Background: Previous reports suggest flavonoids as potent analgesic compounds.

Objectives: Based on these observations, the present study investigated the anti-nociceptive action of troxerutin and neural interactions with opioidergic, serotoninergic, and nitrergic systems in mice.

Methods: A total of 340 male mice were randomly divided into 2 categories. Each category included four experiments with four groups. In the first experiment of formalin examination, the animals intraperitoneally received saline and troxerutin (50, 150, and 300 mg/kg). In the second experiment, the animals received saline, naloxone (2 mg/kg), troxerutin (300 mg/kg), and troxerutin + naloxone. In the third and fourth experiments, L-NAME (*L-NG-Nitroarginine methyl ester)* and cyproheptadine were injected. In this test, formalin was injected and paw licking time (pain sense) was recorded. In the writhing test, experimental groups were treated similarly and the mice were injected with acetic acid. Then, the inhibition of the writhing movements was recorded.

Results: According to the findings, troxerutin decreased pain in the formalin test and writhing movements in the writhing test ($P=0.001$). Naloxone and troxerutin decreased licking time and writhing movements ($P=0.001$). L-NAME + troxerutin significantly diminished the anti-nociceptive effect of troxerutin on paw licking and inhibited pain response ($P=0.001$).

Conclusion: These results suggested that troxerutin decreases inflammatory pain in mice, and this effect is mediated by opioidergic and nitrergic systems.

Keywords: Anti-nociceptive, Troxerutin, Mice

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Background

Pain is a complex perceptual experience resulting from abnormal injury or dysfunction of peripheral nociceptive neurons. Pain is classified as nociceptive, neuropathic, and psychogenic based on stimulus type, size, duration, and severity (1). Inflammatory pain occurs as a results of the release of the inflammatory mediators from injured tissue (2). The opioid system is a primary inhibitory system of nociception in the body. However, serotonin, noradrenaline, dopamine, endocannabinoid, nitric oxide, and several peptides act as nociceptive neurotransmitters (3). Non-steroidal anti-inflammatory drugs (NSAIDs) and opiates are widely used for pain relief, but their side effects still pose a problem (4). Numerous investigations have been done for developing new anti-inflammatory drugs (5).

Natural and synthetic flavonoids have several pharmacological and therapeutic activities including anti-nociceptive and anti-inflammatory (6). Troxerutin, known as vitamin P4, is a derivative of naturally occurring bioflavonoid rutin found in tea, coffee, cereal grains, and various fruits and vegetables. It is water soluble and can be absorbed by the gastrointestinal system without cytotoxic effects. Troxerutin is favorable because of its antioxidant, anti-inflammatory, neuroprotective, and anti-diabetic activities (7). Troxerutin is known to have anti-inflammatory and antioxidant properties (8). It quickly passes through the blood-brain barrier and its administration is associated with lower pyramidal cells with disorganized layers and karyopyknosis in the hippocampus of diabetic rats (7). However, there is no report on the possible effect of troxerutin on pain relief. Therefore, we primarily assessed the possible anti-nociceptive effect of troxerutin using both the formalin

and writhing tests, and secondly its possible interaction with opioidergic, nitrergic, and serotoninergic systems in mice.

Materials and Methods

Animals

In this study, 340 adult male NMRI mice (weighing 25-30 g) were kept in a standard laboratory condition with free access to pellets and water. Animals were randomly allocated into two categories as formalin and writhing tests, each included 4 experiments with 4 groups (n = 10) (9).

Drugs and Chemicals

Troxerutin, morphine, naloxone, nitric oxide inhibitor (L-NAME, *L-NG-Nitroarginine methyl ester*), and serotonergic receptor antagonist (cyproheptadine) were obtained from Sigma (St. Louis, MO, USA). Drugs were first dissolved in saline and then were intraperitoneally injected (volume of 0.5 mL).

Formalin Test

The formalin test was done as previously described by Hunskaar and Hole (10,11). In the first experiment, mice were treated with saline, troxerutin (50 mg/kg), troxerutin (150 mg/kg), troxerutin (300 mg/kg), and morphine (5 mg/kg). Thirty minutes later, formalin 1% solution (50 µL) was injected into the plantar surface of the right paw (Figure 1). In the second experiment, animals were treated with saline, naloxone (2 mg/kg), troxerutin (most effective dose of troxerutin, 300 mg/kg), and troxerutin + naloxone (Figure 2). In the groups with two injections, animals primarily received the antagonist, and after 15 minutes, troxerutin was administrated. Additionally, 15 minutes later, formalin was injected and immediately the pain response was determined as time spent licking and biting the injected paw. In the third experiment, the mice were intraperitoneally injected with saline, L-NAME (10 mg/kg), troxerutin (300 mg/kg), and troxerutin + L-NAME (Figure 3). In the fourth experiment, saline, cyproheptadine (4 mg/kg), troxerutin (300 mg/kg),

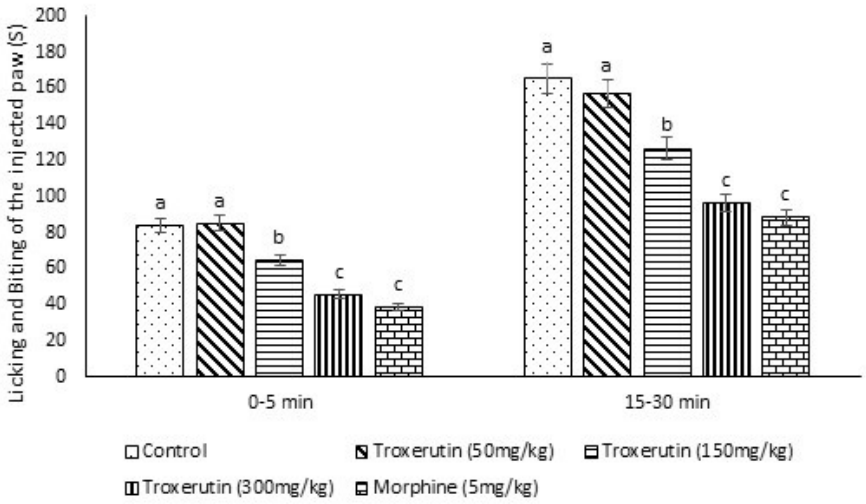

Figure 1. Effect of Troxerutin on Licking and Biting Time of the Injected Paw in Male Mice (n = 50). Data are expressed as mean ± SE. Different superscripts (a-c) indicate significant differences between groups ($P < 0.05$)

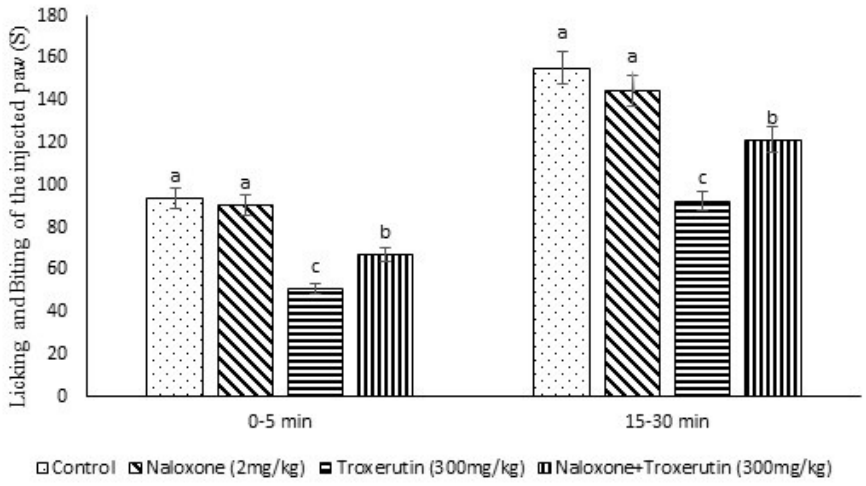

Figure 2. Effect of Naloxone, Troxerutin, and their Co-injection on Licking and Biting Time of the Injected Paw in Male Mice (n = 40). Naloxone: opioid receptor antagonist. Data are expressed as mean ± SE. Different superscripts (a-c) indicate significant differences between groups ($P < 0.05$)

and troxerutin + cyproheptadine were injected (Figure 4).

Writhing Test

Writhing or abdominal contraction appears as a result of the pain which is used for describing inflammatory pain (12,13). The inhibition of pain was determined using the following equation: (control mean – treatment mean) × 100/control mean (12). In the first experiment, mice received saline, troxerutin (50 mg/kg), troxerutin (150 mg/kg), troxerutin (300 mg/kg), and morphine (5 mg/kg). Then, 30 minutes later, the animals received acetic acid (0.6%) and were observed until 30 minutes after the injection (Table 1). In the second experiment, the mice were treated with saline, naloxone (2 mg/kg), troxerutin (most effective dose of troxerutin, 300 mg/kg), and troxerutin + naloxone (Table 2). In groups with 2 injections, the mice first received antagonist, and 15 minutes later, troxerutin (300 mg/kg) was injected. Additionally, 15 minutes later, they were injected with acetic acid the writhing movements were recorded until 30 minutes after the injection. In the third experiment, saline, L-NAME (10 mg/kg), troxerutin (300 mg/kg), and troxerutin + L-NAME were injected (Table 3). In the fourth experiment, the mice were treated with saline, cyproheptadine (4 mg/kg), troxerutin (300 mg/kg), and troxerutin + cyproheptadine (Table 4).

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) and expressed as mean ± standard error (SE) using SPSS version 22.0. For treatments showing significant differences by ANOVA, between group comparisons were done using Tukey post hoc test ($P < 0.05$).

Results

Anti-nociceptive Effects of Troxerutin

As presented in Figure 1 and Table 1, morphine decreased licking and biting time (pain response) in phases I and II of the formalin test and the total number of writhing movements, respectively ($P = 0.001$). As seen, troxerutin decreased pain when compared with control animals ($P = 0.001$).

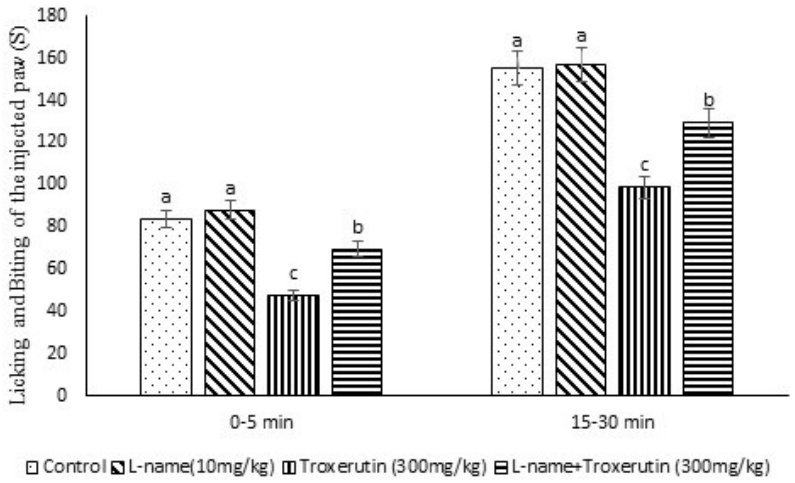

Figure 3. Effect of L-NAME, Troxerutin, and their Co-injection on Licking and Biting Time of the Injected Paw in Male Mice (n = 40). L-NAME: L-NG-Nitro arginine methyl ester, nitric oxide inhibitor. Data are expressed as mean ± SE. Different superscripts (a-c) indicate significant differences between groups ($P < 0.05$)

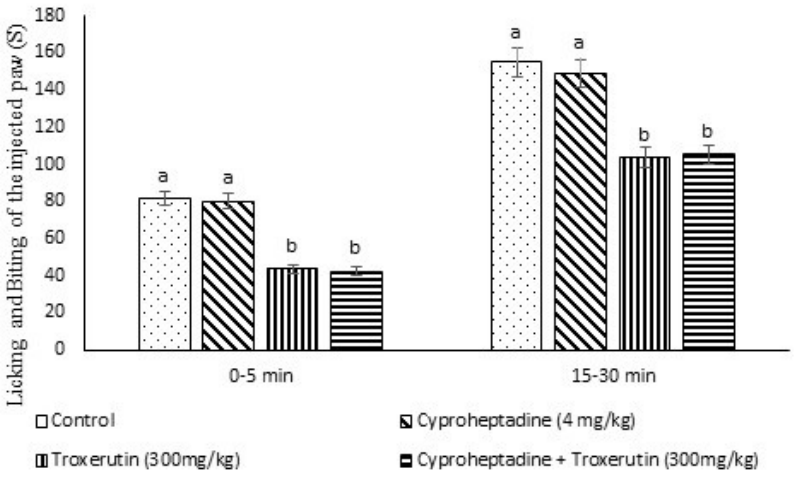

Figure 4. Effect of Cyproheptadine, Troxerutin, and their Co-injection on Licking and Biting Time of the Injected Paw in Male Mice (n = 40). Cyproheptadine: Serotonergic receptor antagonist. Data are expressed as mean ± SE. Different superscripts (a-b) indicate significant differences between the groups ($P < 0.05$)

Table 1. Effect of different doses of troxerutin on acetic acid-induced writhing tests in mice (n = 10)

Experimental groups	Writhing count	Inhibition (%)	*P* value
Control	84.34 ± 3.65	0	0.432
Troxerutin (50 mg/kg)	76.32 ± 3.11	9.50	0.670
Troxerutin (150 mg/kg)	62.13 ± 2.21	21.56 *	< 0.001
Troxerutin (300 mg/kg)	41.23 ± 2.64	51.11 *	< 0.001
Morphine (5 mg/kg)	25.43 ± 2.33	69.84 *	< 0.001

Values are presented as mean ± standard error. *$P < 0.05$ vs. control.

Table 2. Effect of Naloxone, Troxerutin, and Their Co-administration on Acetic Acid-Induced Writhing Test in Mice (n = 10)

Experimental groups	Writhing count	Inhibition (%)	*P* Value
Control	84.64 ± 3.53	0	0.278
Naloxone (2 mg/kg)	75.22 ± 3.44	11.12	0.540
Troxerutin (300 mg/kg)	46.12 ± 2.71	45.51*	< 0.001
Naloxone + Troxerutin	60.34 ± 2.63	28.70*	< 0.001

Naloxone: Opioid receptor antagonist. Values are presented as mean ± standard error. *$P < 0.05$ vs. control.

Table 3. Effect of L-NAME, Troxerutin, and Their Co-administration on Acetic Acid-Induced Writhing Test in Mice (n = 10)

Experimental groups	Writhing count	Inhibition (%)	*P* value
Control	83.14 ± 3.56	0	0.561
L-NAME (10 mg/kg)	77.34 ± 4.68	6.97	0.630
Troxerutin (300 mg/kg)	42.87 ± 2.87	48.43 *	< 0.001
L-NAME + Troxerutin	59.89 ± 2.86	27.96 *	< 0.001

L-NAME: *L-NG-Nitroarginine methyl ester*. Values are presented as mean ± standard error. *$P < 0.05$ vs. control.

Table 4. Effect of Cyproheptadine, Troxerutin, and their Co-administration on Acetic Acid-induced Writhing Test in Mice (n = 10)

Experimental Groups	Writhing Count	Inhibition (%)	*P* Value
Control	85.32 ± 3.23	0	0.612
Cyproheptadine (4 mg/kg)	78.76 ± 3.43	7.68	0.860
Troxerutin (300 mg/kg)	45.67 ± 2.32	46.47 *	< 0.001
Cyproheptadine + Troxerutin	47.32 ± 2.65	44.53	0.710

Cyproheptadine: Serotonergic receptor antagonist. Values are presented as mean ± standard error. *$P < 0.05$ vs. control.

Involvement of the Opioidergic System in Troxerutin-Induced Anti-nociception

According to Figure 2 and Table 2, naloxone (2 mg/kg) did not induce anti-nociceptive response in either test ($P = 0.540$). Troxerutin (300 mg/kg) inhibited pain response in the injected paw (45.51%) when compared with the control mice ($P = 0.001$). In addition, the injection of naloxone + troxerutin diminished pain response in the formalin test and writhing movements (28.70%) in the writhing test when compared with the control animals ($P = 0.001$). It seems that the observed effects of troxerutin were mediated by the opioidergic system.

Involvement of the Nitrergic System in Troxerutin-Induced Anti-nociception

According to Figure 3 and Table 3, L-NAME (10 mg/kg) did not induce anti-nociception in formalin and writhing tests ($P = 0.630$). Troxerutin (300 mg/kg) significantly reduced pain response in injected paw ($P = 0.001$) and writhing movements by 48.43 % in comparison with control animals ($P = 0.001$). Co-administration of L-NAME and troxerutin significantly diminished the anti-nociceptive effect of troxerutin on the pain response of the injected paw in the formalin test and inhibition of pain response (27.96 %) in the writhing test in comparison with control mice ($P = 0.001$). It seems that some anti-nociceptive effects of troxerutin were mediated by the nitrergic system.

Involvement of the Serotoninergic System in Troxerutin-Induced Anti-nociception

According to Figure 4 and Table 4, there was no anti-nociceptive effect following the administration of cyproheptadine (4 mg/kg) ($P = 0.860$). Troxerutin (300 mg/kg) hindered pain response in injected paw ($P = 0.001$) and writhing movements (46.47%) in comparison with control mice ($P = 0.001$). The co-injection of cyproheptadine and troxerutin affected the anti-nociceptive activity of troxerutin using formalin and writhing tests (44.53% inhibition of the writhing movements) ($P = 0.710$). It seems that the serotoninergic system has no role in the anti-nociceptive effects of troxerutin.

Discussion

This study was designed to investigate the possible modulatory effect of troxerutin on pain using formalin and writhing tests in mice (14). Injection of formalin into hind paw or acetic acid into the intraperitoneal cavity leads to the release of pro-inflammatory mediators, which can induce pain by activating peripheral nociceptors (15). Morphine, at an effective dose, decreased the pain response and troxerutin showed similar activity using formalin and writhing tests. Scarce information exists on the anti-nociceptive activity of troxerutin. Troxerutin suppresses the activation of NF-κB signaling in neuropathic pain (14). Troxerutin has an anti-allodynic effect via the AMPK/SIRT1 pathway in CCI-induced neuropathic pain (16).

In this study, the co-injection of naloxone and troxerutin diminished pain response. Naloxone blocked the anti-nociception in formalin-induced paw licking time in hot plate tests (17). Opioidergic receptors are expressed in the spinal cord, nucleus raphe magnus, and the periaqueductal gray (18). Besides, the co-administration of L-NAME and troxerutin diminished the anti-nociceptive effect of troxerutin. Intraplantar injection of the formalin increased nitric oxide and TNF-α gene expression, and pre-treatment with L-NAME reversed it (19). Nitric oxide is an oxidant, and its level is elevated after neuropathic sciatic pain (20). Nitric oxide as a signaling molecule plays an essential role in inflammatory response (21). Nitric oxide has a vital nociceptive activity in central and peripheral nervous systems. Additionally, it increases the synthesis or release of reactive oxygen species in inflammatory pain (15). This

naturally occurring compound has antioxidant and anti-inflammatory properties and can decrease oxidative stress and neuropathic pain (22). In this regard, Dadpisheh et al (23) reported that troxerutin improves levels of catalase, paraoxonase 1, glutathione peroxidase, and nitric oxide in sciatic nerve ischemia-reperfusion injury. Oxidative stress, resulting in an imbalance between the production of oxygen free radicals and antioxidant capacity, damages the biological macro-molecules and disrupts normal metabolism and physiology (22). Endogenous antioxidants exert their activity by scavenging oxygen free radicals, thereby delaying or inhibiting cellular damage mainly through their free radical scavenging property. Moreover, troxerutin scavenges reactive oxygen species and decreases NF-κB expression in diabetic rats (24) or patients suffering from cardiovascular diseases (22).

However, due to the limitations, we could not define serum activity of antioxidants (superoxide dismutase, glutathione peroxidase, catalase) or nitric oxide following the administration of troxerutin in formalin and acetic acid-injected mice. These results suggest that troxerutin has anti-nociceptive effect, which is mediated by opioidergic and nitrergic systems in mice.

Conclusion

These results suggested that troxerutin decreases inflammatory pain in mice, and this effect is mediated by opioidergic and nitrergic systems.

Acknowledgments

This paper is related to DVM thesis of the first author .

Competing Interests

The authors reported no potential conflict of interest.

Ethical Approval

All experimental procedures approved at the Animal Ethics Committee of the Science and Research Branch of Islamic Azad University, Tehran, Iran (IR.IAU.SRB.REC.1401.30).

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