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

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 result 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 serotoninergic 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](image1.png)

**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](image2.png)

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

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Writhing count</th>
<th>Inhibition (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>84.34 ± 3.65</td>
<td>0</td>
<td>0.432</td>
</tr>
<tr>
<td>Troxerutin (50 mg/kg)</td>
<td>76.32 ± 3.11</td>
<td>9.50</td>
<td>0.670</td>
</tr>
<tr>
<td>Troxerutin (150 mg/kg)</td>
<td>62.13 ± 2.21</td>
<td>21.56</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Troxerutin (300 mg/kg)</td>
<td>41.23 ± 2.64</td>
<td>51.11</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Morphine (5 mg/kg)</td>
<td>25.43 ± 2.33</td>
<td>69.84</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

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)

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Writhing count</th>
<th>Inhibition (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>84.64 ± 3.53</td>
<td>0</td>
<td>0.278</td>
</tr>
<tr>
<td>Naloxone (2 mg/kg)</td>
<td>75.22 ± 3.44</td>
<td>11.12</td>
<td>0.540</td>
</tr>
<tr>
<td>Troxerutin (300 mg/kg)</td>
<td>46.12 ± 2.71</td>
<td>45.51</td>
<td>&gt; 0.001</td>
</tr>
<tr>
<td>Naloxone + Troxerutin</td>
<td>60.34 ± 2.63</td>
<td>28.70</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

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)

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Writhing count</th>
<th>Inhibition (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>83.14 ± 3.56</td>
<td>0</td>
<td>0.561</td>
</tr>
<tr>
<td>L-NAME (10 mg/kg)</td>
<td>77.34 ± 4.68</td>
<td>6.97</td>
<td>0.630</td>
</tr>
<tr>
<td>Troxerutin (300 mg/kg)</td>
<td>42.87 ± 2.87</td>
<td>48.43</td>
<td>&gt; 0.001</td>
</tr>
<tr>
<td>L-NAME + Troxerutin</td>
<td>59.89 ± 2.86</td>
<td>27.96</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

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)

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Writhing Count</th>
<th>Inhibition (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85.32 ± 3.23</td>
<td>0</td>
<td>0.612</td>
</tr>
<tr>
<td>Cyproheptadine (4 mg/kg)</td>
<td>78.76 ± 3.43</td>
<td>7.68</td>
<td>0.860</td>
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<tr>
<td>Troxerutin (300 mg/kg)</td>
<td>45.67 ± 2.32</td>
<td>46.47</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cyproheptadine + Troxerutin</td>
<td>47.32 ± 2.65</td>
<td>44.53</td>
<td>0.710</td>
</tr>
</tbody>
</table>

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 Nitric 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 nitricergic system.

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 using formalin and writhing tests. It seems that the serotoninergic system has no role in the anti-nociceptive effects of troxerutin.
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-kB 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.

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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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References
Anti-nociceptive effect of troxerutin in mice


