Antinociceptive Properties of Ascorbic Acid: Evidence for the Mechanism of Action

Fatemeh Zeraati \(^1\); Malihe Araghchian \(^1\); Farzaneh Esna-ashari \(^2\); Mohammad Mehdi Fazlian \(^3\); Sima Torabian \(^3\); Nazanin Fallah \(^3\); Marjan Ghavimi \(^3\)

\(^1\)Department of Pharmacology, Hamadan University of Medical Sciences, Hamadan, IR Iran
\(^2\)Department of Community Medicine, Hamadan University of Medical Sciences, Hamadan, IR Iran
\(^3\)Medical school, Hamadan University of Medical Sciences, Hamadan, IR Iran

*Corresponding author: Fatemeh Zeraati, Department of Pharmacology, Hamadan University of Medical Sciences, Hamadan, IR Iran. Tel: +98-9183122063, Fax: +98-8118226035, E-mail: zeraati@umsha.ac.ir

Received: March 28, 2014; Accepted: April 21, 2014

Background: Ascorbic acid is amongst important water-soluble vitamins and when used orally in high-doses it has been observed to relieve pain and reduce opioid use in patients. However no controlled trial has compared the antinociceptive effects of ascorbic acid with other analgesic groups on animal models, and investigated the involved mechanisms.

Objectives: In the present study, the antinociceptive effect of vitamin C on male mice was investigated and compared with morphine and diclofenac. Also, possible mechanisms were assayed.

Materials and Methods: Male albino mice were used in this study. Antinociception was measured using the writhing test, tail flick and formalin tests. Ascorbic acid was used in three doses (30, 150 and 300 mg/kg, IP) and compared with the antinociceptive effects of 10 mg/kg of morphine as an opioid analgesic agent and 5-10 mg/kg of diclofenac as a nonsteroidal anti-inflammatory drug (NSAID) analgesic agent. The antinociceptive effect of ascorbic acid (300 mg/kg) was compared before and after treatment with naloxone (4 mg/kg), ondansetron (0.5 mg/kg), atropine (5 mg/kg) and metoclopramide (1 mg/kg) in the writhing test.

Results: Vitamin C caused dose-dependent antinociceptive effects in acetic acid writhing test \((P < 0.05)\). It had no significant effect in the tail flick test. Meanwhile, vitamin C in high doses reduced pain in the second phase of the formalin test \((P < 0.05)\). Morphine had higher nociceptive effects in comparison to ascorbic acid in the writhing test \((P < 0.05)\). In the second phase of the formalin test the antinociceptive effects of vitamin C (300 mg/kg) was not significantly different with morphine at dose of 10 mg/kg. There was not significant difference between vitamin C (300 mg/kg) and diclofenac (10 mg/kg) in the second phase of the formalin test. Metoclopramide and ondansetron reduced the antinociceptive effects of vitamin C.

Conclusions: The results obtained from the acetic acid induced writhing test and second phase of the formalin test indicate that vitamin C possess antinociceptive activity especially on inflammatory pain. Ondansetron and metoclopramide reduced the effects of ascorbic acid, which may be because ascorbic acid produced antinociception through mechanisms that may be involved in dopaminergic and serotonergic systems.

Keywords: Ascorbic Acid; Nociception; Diclofenac; Morphine; Dopaminergic; Cholinergic Agents

1. Background

Ascorbic acid (vitamin C) is amongst important water-soluble vitamins. It is essential for collagen, carnitine and neurotransmitters biosynthesis. Most plants and animals synthesize vitamin C for their own requirements. However humans cannot synthesize ascorbic acid due to the lack of an enzyme called gulonolactone oxidase. Many health benefits have been attributed to ascorbic acid such as antioxidant, antiatherogenic, anticarcinogenic and immunomodulator effects as well as its ability to prevent cold, etc. (1).

It is a powerful antioxidant and at physiological concentrations, it probably does not produce reactive intermediaries. It protects low-density lipoproteins from oxidation, reduces harmful oxidants in the stomach and promotes iron absorption (2). Pain relieving effects of mega doses of ascorbate were reported many years ago by Dr. Klenner for the treatment of severe burns and snake bites in human (3). Ascorbic acid, when consumed orally in high doses, has been observed to relieve pain and reduce opioid use in cancer patients (4). Daily administration of vitamin C reduced arthritic swelling and increased pain tolerance in rat paws (5).

Implication for health policy/practice/research/medical education:
This study compared the antinociceptive effects of ascorbic acid with other analgesic groups on animal models and investigated some of the mechanism that may be involved in this antinociceptive system.
2. Objectives
In the present study, the antinociceptive effects of vitamin C in the male mice were investigated and compared with morphine and diclofenac. Also, the possible mechanisms of action of vitamin C were assayed.

3. Materials and Methods

3.1. Animals
In total, 121 male albino NMRI (Naval Medical Research Institute) mice (institute of the Pasteur, Tehran) weighing 25-32 g were used in these experiments. All experiments were performed in accordance with the institutional animal use guidelines. The animals were housed in standard stainless steel cages in a temperature controlled room (22 ± 2°C) with a 12-12 hours light-dark cycle. Mice were randomly distributed into groups of 6-10 as control and test subjects. All animals had access to food and water throughout the experiments. For antinociception recording, mice were allowed to acclimatize for 30 minutes before any injection.

3.2. Drugs
The chemicals used were: ascorbic acid, acetic acid and formalin (Merck, Germany), diclofenac (Merck, Germany), morphine sulfate and atropine (Darul Pakhsh, Iran), naloxone (Toliddaru, Iran), ondansetrone (Exir, Iran) and metoclopramide (Osveh, Iran).

3.3. Writhing Test
For this test, 0.1 mL/10g body weight of an aqueous acetic acid solution (0.7% V/V) was administered by intraperitoneal injection and the number of abdominal contractions was counted for 10 minutes, five minutes post-injection (6). An intraperitoneal injection of ascorbic acid was performed, 30 minutes before acetic acid and other drugs were injected IP, 20 minutes before ascorbic acid.

3.4. Tail Flick Test
Antinociception was assessed using the tail flick test. The tail withdrawal latency (second) was measured before administration of any drug or vehicle. Normal response latencies were usually between 2.5 and 3.0 seconds and a ten-second cut-off was used to prevent tissue damage. The response was tested 10, 40, 70 and 100 minutes after drug administration. Antinociception was quantified as the percentage of maximum possible effect (MPE%) using the method of Keil and Delander (7). The following formula was used to calculate MPE%: MPE% = 100 × [(test-control latency)/(cutoff-control latency)].

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>Number of Writhes, Mean ± SD</th>
<th>Number of Writhes, Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>10</td>
<td>32.8 ± 2.6</td>
<td>32.8 ± 2.6</td>
</tr>
<tr>
<td>Morphine</td>
<td>6</td>
<td>0.1 ± 0</td>
<td>0.1 ± 0</td>
</tr>
<tr>
<td>Vitamin C, 30 mg</td>
<td>7</td>
<td>28.9 ± 2.2</td>
<td>28.9 ± 2.2</td>
</tr>
<tr>
<td>Vitamin C, 150 mg</td>
<td>7</td>
<td>21 ± 2.3</td>
<td>21 ± 2.3</td>
</tr>
<tr>
<td>Vitamin C, 300 mg</td>
<td>7</td>
<td>10.9 ± 2.3</td>
<td>10.9 ± 2.3</td>
</tr>
</tbody>
</table>

Table 2. The Effects of Ascorbic Acid (Vitamin C) Administration (IP) and Morphine According to the Writhing Test

<table>
<thead>
<tr>
<th>No.</th>
<th>Saline</th>
<th>Morphine</th>
<th>Vitamin C, 30 mg</th>
<th>Vitamin C, 150 mg</th>
<th>Vitamin C, 300 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPE%, Pre-drug</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| No. | 10 | 7 | 7 | 7 | 7 |
| MPE, post-drug, min | 2 ± 0.9 | 4.87 ± 1.96 | 1.94 ± 1.3 | 5.97 ± 1.4 | 5.2 ± 0.6 |
| 40 | 3.1 ± 0.29 | 43.64 ± 12.3 | 3.27 ± 0.76 | 1.76 ± 0.79 | 4.6 ± 1.3 |
| 70 | 1.3 ± 0.4 | 30.17 ± 12.69 | 2.7 ± 0.8 | 4.97 ± 0.9 | 4.28 ± 0.7 |
| 100 | 3.2 ± 1.3 | 17.71 ± 6.03 | 3.6 ± 1.4 | 1.6 ± 1.8 | 4.1 ± 1.4 |

Abbreviation: MPE, maximum possible effect.
Data are presented as Mean ± SEM.
The differences in responses were analyzed by ANOVA and followed by the Newman-Keuls test.
P < 0.05 compared with control.
Table 3. The Effects of Ascorbic Acid (Vitamin C) Administration (IP) and Morphine on the Formalin Test a,b

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>First Phase Licking Time, s</th>
<th>Second Phase Licking Time, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>8</td>
<td>114.3 ± 10</td>
<td>133.7 ± 27</td>
</tr>
<tr>
<td>Morphine</td>
<td>6</td>
<td>26.8 ± 8 b</td>
<td>2.5 ± 2 b</td>
</tr>
<tr>
<td>Vitamin C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 mg</td>
<td>6</td>
<td>101 ± 14</td>
<td>142.4 ± 31</td>
</tr>
<tr>
<td>150 mg</td>
<td>6</td>
<td>105.8 ± 9</td>
<td>101.2 ± 30</td>
</tr>
<tr>
<td>300 mg</td>
<td>6</td>
<td>104.8 ± 12 b</td>
<td>30.2 ± 7 b</td>
</tr>
</tbody>
</table>

a Data are presented as Mean ± SEM.

b P < 0.05 compared with control.

3.5. Formalin Test

The formalin test was performed as described by Hunskaar et al. (8). Primarily, 25 µL of 2.5% (V/V) formalin in saline was injected into the dorsal surface of the right hind paw. Immediately after the formalin injection, animals were placed individually in a glass cylinder (20 cm wide and 30 cm long) on a flat glass floor and a mirror was arranged at an angle of 90 degrees under the cylinder to allow clear observation of the paws.

Care was taken to exclude environmental disturbances (high temperature, noise and excessive movement) that might interfere with the animal’s response. Only licking or biting of the injected paw was defined as a nociceptive response. The total time of responses was measured during periods of 0-5 minutes (first phase) and 20-40 minutes (second phase). All animals were brought to the test chamber 30 minutes prior to the experiments. The mice were not tested more than once and testing took place between 09:00 am and 12:00 am.

3.6. Statistical Analysis

One way ANOVA and the Newman-Keuls and Dunnett’s post hoc tests were used to analyze the data. Differences between means with P < 0.05 were considered statistically significant. Each point represents the mean ± standard error of the mean (SEM) of the recording for mice in each group.

4. Results

Effect of ascorbic acid and morphine from the writhing test: Intraperitoneal administration of ascorbic acid (150 and 300 mg/kg) induced dose-dependent antinociception (Table 1). In animals that received vitamin C (150 or 300 mg/kg), the number of contractions was signifi-
cantly altered relative to controls (P < 0.01). The number of contractions in animals that received vitamin C (300 mg/kg) was significantly different relative to morphine (10 mg/kg). Morphine recipients experienced higher antinociceptive effects in comparison to ascorbic acid.

4.1. Pretreatment With Drugs in the Writhing Test
Effect of ascorbic acid and morphine on tail flick test: Ascorbic acid (30, 150 and 300 mg/kg) had no effect in the tail flick test (Table 2).

Effect of ascorbic acid and morphine in the formalin test: During the first phase (0-5 minutes after formalin injection) only morphine induced a significant alteration. In the second phase of the formalin test acetic acid at a dose of 300 mg/kg and morphine at a dose of 10 mg/kg, significantly reduced the period that mice spent licking their paws (P < 0.05). In the second phase, this time period in animals that had received vitamin C (300 mg/kg) was not significantly different from those that received morphine at dose of 10 mg/kg (Table 3).

Pretreatment with naloxone and atropine could not change antinociceptive effects of ascorbic acid (300 mg/kg) (P > 0.05). Metoclopramide and ondansetron reduced ascorbic acid (300 mg/kg) effect and increased writhing number in comparison with the group that received ascorbic acid (P < 0.05) (Figure 1).

The licking time was significantly reduced in the second phase of the formalin test (P < 0.001) with diclofenac and vitamin C injection. However, in comparison with the control group, ascorbic acid didn’t have analgesic effects in the first phase of the formalin test (P > 0.05). There was no significant difference between vitamin C and diclofenac groups in the second phase of the formalin test (P > 0.05). The combination of vitamin C and diclofenac significantly reduced licking time in the first phase (P = 0.000) (Figures 2 and 3).

5. Discussion
The present study investigated the antinociceptive responses to vitamin C in male mice using acetic acid induced writhing test, tail flick and formalin test, and compared these responses to that caused by morphine and diclofenac administration. In the writhing test, different doses of vitamin C (150 and 300 mg/kg) induced antinociception in a dose dependent manner. Although abdominal writhes induced by acetic acid represent a peripheral nociception model (9), this is not a specific model, since several compounds, such as, tricyclic antidepressants (10) and anti-histamine (11) inhibit the writhes induced by acetic acid. Writhes induced by IP injections of acetic acid are said to originate from the pain of inflammation mediated by prostaglandins (12, 13). It has been shown that vitamin C inhibits the COX-2 enzyme activity and acts synergistically with acetyl salicylic acid in inhibiting PGE2 (14, 15).

The tail-flick test is widely used to investigate central analgesic activity. The tail-flick response appears to be a spinal reflex, which is modulated by a supra spinal inhibitory mechanism (16). Vitamin C had no effect in this test. This may be because ascorbic acid has no central antinociceptive effects. The formalin test is one of the most used models to explain pain and analgesia mechanisms, with better results than tests using mechanical or thermal stimulus (17). This model constitutes two distinct phases. The first phase represents the irritating effect of formalin at the sensorial fiber-C. The second phase is an inflammatory pain response. Central acting analgesics, such as morphine, inhibit both phases. Peripheral acting drugs, such as non-steroid anti-inflammatory and corticosteroids, inhibit only the second phase (18). The first phase of the formalin test is not pronouncedly altered by different doses of vitamin C, but the second phase, was affected by vitamin C at dose of 300 mg/kg, which was not significantly different from effects induced by morphine (10 mg/kg). Antinociception in the second phase of the formalin test and nocuous stimulation of this phase is attributed to inflammatory activity and/or alteration of central processing (19). Jensen (20) showed that calcium ascorbate reduced pain from osteoarthritis of hip joint or knee joint. Administration of 150 mg/kg of vitamin C reduced arthritic swelling, increased pain tolerance and decreased polymorphonuclear leukocyte infiltration (5).

Ascorbic acid therapy, with high doses, reduced bone pain in Paget’s disease (21). Vitamin C (300 mg/kg) and diclofenac (5 mg/kg) had no antinociceptive effect in the first phase, but the combination of vitamin C (300 mg/kg) and diclofenac (5 mg/kg) resulted a significant effect. This combination could affect acute pain, which was not affected by these drugs alone at the examined doses. Further studies are required to understand the mechanisms of these findings.

Vitamin C had similar analgesic effects as that of diclofenac in the second phase of the formalin test. Antinociceptive effect of ascorbic acid was not antagonized by atropine and naloxone. Several reports support a role for acetylcholine (ACh) in the inhibition and modulation of nociceptive information transmission (22, 23). A possibility is that involvement of opioidergic and cholinergic system is canceled.

Studies have shown the involvement of the dopaminergic system in mechanisms of antinociception (24). For instance, dopamine receptors agonists were found to facilitate analgesic responses (25, 26). The present results showed that the antinociceptive action of ascorbic acid was attenuated by metoclopramide.

Pivotal studies have shown the spinal analgesic actions of 5-HT released from brainstem structures (27-29). In this study, the analgesic effect of ascorbic acid was inhibited by ondansetron, a serotonin antagonist. Attenuation of antinociceptive action of vitamin C by ondansetron in mice indicates that serotonin receptors play an important role in the modulation of pain perception by serotonin.
In conclusion, ascorbic acid showed peripheral antinociceptive action, which is likely to involve anti-inflammatory mechanisms and the involvement of dopaminergic and serotoninergic system can be important.

Acknowledgements

This manuscript was extracted from the thesis of Dr. Mohammad Mehdi Fazlian and Sima Torabian (No: 84033113153) and a study (No: 890713121417) that was financially supported by the Vice-Chancellor for Research, Hamadan University of Medical Sciences, Hamadan, Iran. The authors would like to thank the Vice-Chancellor for Research of Hamadan University of Medical Sciences.

Authors’ Contributions

Study concept and design: Dr. Zeraati and Dr. Araghchi-an; acquisition of data: Mohammad Mehdi Fazlian, Sima Torabian, Naznin Fallah and Marjan Ghvimii; analysis and interpretation of data and statistical analysis: Farzaneh Esna-ashari; drafting of the manuscript: Dr. Zeraati.

Funding/Support

This study was financially supported by the Vice-Chancellor for Research, Hamadan University of Medical Sciences, Hamadan, Iran.

References