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

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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 ondansetrone 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. Ondansetrone 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 serotoninergic 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 (Daru 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 1. The Effects of Ascorbic Acid (Vit C) Administration (IP) and Morphine According to the Writhing Test [a]

Groups	No.	Number of Writhes, Mean ± SD
Saline	10	32.8 ± 2.6
Morphine	6	0.1 ± 0
Vitamin C		
30 mg	7	28.9 ± 2.2
150 mg	7	21 ± 2.3 [b]
300 mg	7	10.9 ± 2.3 [c]

[a] The differences in responses were analyzed by ANOVA and followed by the Newman-Keuls test.
[b] P < 0.01 compared with control.
[c] P <0.05 compared with Vitamin C 150 mg.

Table 2. The Effect of Ascorbic acid (Vitamin C) Administration (IP) and Morphine on the Tail Flick Test [a,b,c]

	Saline	Morphine	Vitamin C, 30 mg	Vitamin C, 150 mg	Vitamin C, 300 mg
No.	10	7	7	7	7
MPE%, Pre-drug	0	0	0	0	0
MPE, post-drug, min					
10	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 [d]	3.27 ± 0.76	1.76 ± 0.79	4.6 ± 1.3
70	1.3 ± 0.4	30.17 ± 12.69 [d]	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

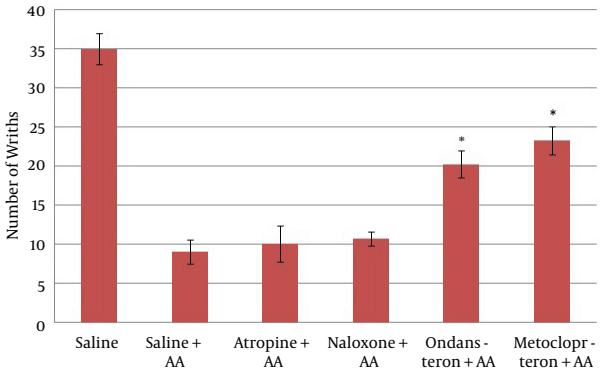
[a] Abbreviation: MPE, maximum possible effect.
[b] Data are presented as Mean ± SEM.
[c] The differences in responses were analyzed by ANOVA and followed by Newman-Keuls test.
[d] P < 0.05 compared with control.

Table 3. The Effects of Ascorbic Acid (Vitamin C) Administration (IP) and Morphine on the Formalin Test [a,b]

Groups	No.	First Phase Licking Time, s	Second Phase Licking Time, s
Saline	8	114.3 ± 10	133.7± 27
Morphine	6	26.8 ± 8 [b]	2.5 ± 2 [b]
Vitamin C			
30 mg	6	101 ± 14	142.4 ± 31
150 mg	6	105.8 ± 9	101.2 ± 30
300 mg	6	104.8 ± 12	30.2 ± 7 [b]

[a] Data are presented as Mean ± SEM.
[b] P < 0.05 compared with control.

Figure 1. Comparison Between Ascorbic Acid (300 mg/kg) Antinociceptive Effects Before and After Treatment With Atropine (5 mg/kg), Ondansetrone (0.5 mg/kg), Naloxone (4 mg/kg) and Metoclopramide (1 mg/kg) in the Writhing Test



Each column shows Mean± SEM of writhes in 7-10 mice.

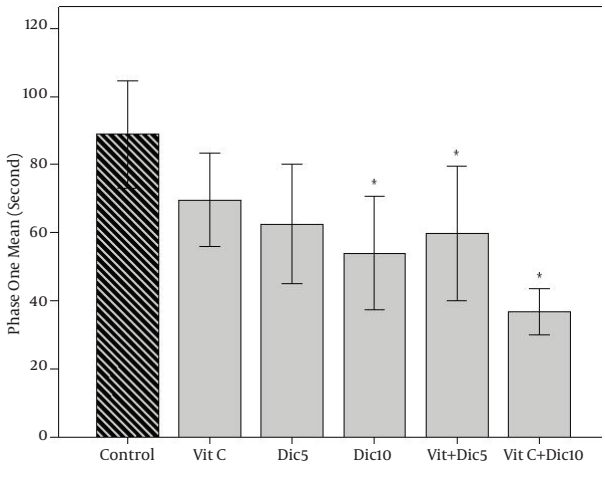


Figure 2. Comparison Between Ascorbic Acid (300 mg/kg), Diclofenac (5 & 10 mg/kg) and Their Combination in the First Phase of the Formalin Test, P < 0.05

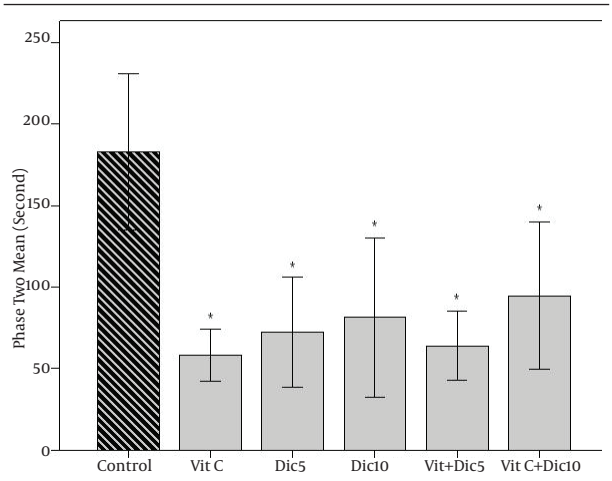


Figure 3. Comparison Between Vitamin C (300 mg/kg), Diclofenac (5 & 10 mg/kg) and Their Combination in the Second Phase of the Formalin Test, P < 0.05

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 ascorbic 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 recieved 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 ondansetrone 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 diclophenac 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 noxious 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 diclofanac (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 effectsas that of diclofenac in the second phase of the formalin test. Antinociception 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.

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Authors' Contributions

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

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References

1. Naidu KA. Vitamin C in human health and disease is still a mystery? An overview. *Nutr J.* 2003;**2**:7.
2. Padayatty SJ, Levine M. New insights into the physiology and pharmacology of vitamin C. *CMAJ.* 2001;**164**(3):353–5.
3. Klenner FR. Significance of high daily intake of ascorbic acid in preventive medicine. *J Intra Acad Prev Med.* 1974;**1**(1):45–69.
4. Evangelou A, Kalfakakou V, Georgakas P, Koutras V, Vezyraki P, Iliopoulou L, et al. Ascorbic acid (vitamin C) effects on withdrawal syndrome of heroin abusers. *In Vivo.* 2000;**14**(2):363–6.
5. Davis RH, Rosenthal KY, Cesario LR, Rouw GR. Vitamin C influence on localized adjuvant arthritis. *J Am Podiatr Med Assoc.* 1990;**80**(8):414–8.
6. Atamer-Simsek S, Olmez-Salvarli H, Guc O, Eroglu L. Antinociceptive effect of amikacin and its interaction with morphine and naloxone. *Pharmacol Res.* 2000;**41**(3):355–60.
7. Keil GJ, 2nd, Delander GE. Time-dependent antinociceptive interactions between opioids and nucleoside transport inhibitors. *J Pharmacol Exp Ther.* 1995;**274**(3):1387–92.
8. Hunskaar S, Fasmer OB, Hole K. Formalin test in mice, a useful technique for evaluating mild analgesics. *jneurosci.* 1985;**14**(1):69–76.
9. Wei ET, Kiang JG, Buchan P, Smith TW. Corticotropin-releasing factor inhibits neurogenic plasma extravasation in the rat paw. *J Pharmacol Exp Ther.* 1986;**238**(3):783–7.
10. Takahashi RN, Paz MM. Influence of naloxone on analgesic effects of antidepressants in mice. *Braz J Med Biol Res.* 1987;**20**(5):607–10.
11. Yeh SY. Potentiation of pentazocine antinociception by tripelennamine in the rat. *J Pharmacol Exp Ther.* 1985;**235**(3):683–9.
12. Gyires K, Knoll J. Inflammation and writhing syndrome inducing effect of PGE1, PGE2 and the inhibition of these actions. *Pol J Pharmacol Pharm.* 1975;**27**(3):257–64.
13. Collier HO, Dinneen LC, Johnson CA, Schneider C. The abdominal constriction response and its suppression by analgesic drugs in the mouse. *Br J Pharmacol Chemother.* 1968;**32**(2):295–310.
14. Fiebich BL, Lieb K, Kammerer N, Hull M. Synergistic inhibitory effect of ascorbic acid and acetylsalicylic acid on prostaglandin E2 release in primary rat microglia. *J Neurochem.* 2003;**86**(1):173–8.
15. Lopez-Lluch G, Fernandez-Ayala DJ, Alcain FJ, Buron MI, Quesada JM, Navas P. Inhibition of COX activity by NSAIDs or ascorbate increases cAMP levels and enhances differentiation in 1alpha,25-dihydroxyvitamin D3-induced HL-60 cells. *Arch Biochem Biophys.* 2005;**436**(1):32–9.
16. Trongsakul S, Panthong A, Kanjanapothi D, Tuesotikul T. The analgesic,antipyretic and anti-inflammatory activity of diospyros variegate kruz. *Ethn.* 2003;**85**(2-3):221–5.
17. Tjølsen A, Berge O, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. *Pain.* 1992;**51**(1):5–17.
18. Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain.* 1987;**30**(1):103–14.
19. Shibata M, Ohkubo T, Takahashi H, Inoki R. Modified formalin test: characteristic biphasic pain response. *Pain.* 1989;**38**(3):347–52.
20. Jensen NH. [Reduced pain from osteoarthritis in hip joint or knee joint during treatment with calcium ascorbate. A randomized, placebo-controlled cross-over trial in general practice]. *Ugeskr Laeger.* 2003;**165**(25):2563–6.
21. Basu TK, Smethurst M, Gillett MB, Donaldson D, Jordan SJ, Williams DC, et al. Ascorbic acid therapy for the relief of bone pain in Paget's disease. *Acta Vitaminol Enzymol.* 1978;**32**(1-4):45–9.
22. Eisenach JC. Muscarinic-mediated analgesia. *Life Sciences.* 1999;**64**(6-7):549–54.
23. Yalcin I, Charlet A, Cordero-Erausquin M, Tessier LH, Picciotto MR, Schlichter R, et al. Nociceptive thresholds are controlled through spinal beta2-subunit-containing nicotinic acetylcholine receptors. *Pain.* 2011;**152**(9):2131–7.
24. Onozawa K, Yagasaki Y, Izawa Y, Abe H, Kawakami Y. Amygdala-prefrontal pathways and the dopamine system affect nociceptive responses in the prefrontal cortex. *BMC Neurosci.* 2011;**12**:115.
25. Michael-Titus A, Bousselmame R, Costentin J. Stimulation of dopamine D2 receptors induces an analgesia involving an opiodergic but non enkephalinergic link. *Eur J Pharmacol.* 1990;**187**(2):201–7.
26. Suaudeau C, Costentin J. Analgesic effect of the direct D2 dopamine receptor agonist RU 24926 and cross tolerance with morphine. *Fundam Clin Pharmacol.* 1995;**9**(2):147–52.
27. Yaksh TL, Tyce GM. Microinjection of morphine into the periaqueductal gray evokes the release of serotonin from spinal cord. *Brain Res.* 1979;**171**(1):176–81.
28. Yaksh TL, Wilson PR. Spinal serotonin terminal system mediates antinociception. *J Pharmacol Exp Ther.* 1979;**208**(3):446–53.
29. Zeitz KP, Guy N, Malmberg AB, Dirajlal S, Martin WJ, Sun L, et al. The 5-HT3 subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors. *J Neurosci.* 2002;**22**(3):1010–9.