Original article

The role of opioids, serotonergic and cholinergic pain receptors to induce analgesic effect of *Trachyspermum ammi* essence using formalin test in mice.

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Abstract

Objective: In previous studies it has been demonstrated that different substances, can inhibit pain occurred through opioids, serotonergic, and cholinergic pain receptors these receptors can be inhibited while in presence of naloxone, ondansetron and atropine. Therefore the aim of this study was to determine the effect of *Trachyspermum ammi* essence on modulation of pain score by the use of formalin test in mice.

Methods: In this experimental study, 20 male mice (20-25) were included. They were randomly divided into test and control groups. The test groups were intraperitoneal injected specific amount of inhibitors such as naloxone, ondansetron and atropine. After 10 min these specimens were injected *Trachyspermum ammi* essence and were observed for 60 min.to score pain effect caused by formalin injection.

Results: Our result indicated that mean score of the pain during the observation for 60 min (every 5 minutes) of formalin test in groups that received ondansetron, naloxone and TAE it is significantly decrease the pain score whiles group of atropine which induced before TAE significantly ($P<0.05$) increased the pain score.

Conclusion: In conclusion, this research demonstrated the possible role of cholinergic signaling in antinociceptive effect. Thus more studies are required to prove the further effects of this essence on different signaling systems.

Keywords: *Trachyspermum ammi Essense, cholinergic receptor,serotonergic receptor, formalin test,pain.*

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Introduction

The analgesic effects of various substances apply through different receptors, including opioid receptors, serotonergic and cholinergic, which inhibits mainly tonic and persistent pain in the cerebral cortex (1), Brain Stem (2) and in the spinal cord (3). Different analgesic receptors attend in areas of the brain and spinal cord mainly involved with transmission and regulation of pain. These receptors, including opioid receptors, serotonergic and cholinergic are kept respectively by naloxone, ondansetron and atropine (4).

Trachyspermum ammi fruit reduce acute pain in mice (5). In addition, alcoholic extracts of trachyspermum ammi reduce chronic pain and has a significantly reduction in the inflammatory phase of formalin-induced pain that may have been due to the essential oils contained in the extract (6). While the non-oil part of trachyspermum ammi extract was not effective on chronic pain in mice and the

reduction in pain compared to the control group is not significant, so according to the analgesic effect of extracts and essential oils, and no analgesic effect in this part may be caused by lack of oil (7).
Findings also showed that trachyspermum ammi oil cause significantly reduction in the inflammatory phase of formalin-induced pain that this effect may be due to the essential oil of trachyspermum ammi, which has analgesic and anti-inflammatory effect Which suggests that this effect may be applied through the cholinergic mechanisms(8). Essential oils from aromatic plants affects on long and double radicals of products (9). Essential oils also inhibit hepatic metabolizing enzyme (1-2 dimethyl hydrazine) that is responsible for hepatic carcinoma (10). Essential oils reduce the release of calcium in sarcoplasmic reticulum and affect on the contractility of the intestine that is caused by acetylcholine (11).
Studies done on this herb show that the extract has anti-histamine, antitussive and relaxant and effects (12, 13, 14), So that relaxant effect is because of essential oil, but not through anti-cholinergic and Beta-adrenergic mechanism. Other studies (15) indicate anti-bacterial effects of essential oil. On the other hand, anti-worm effects of trachyspermum ammi that it is related to thymol compounds and anti-spasmodic and anti flatulence of essential oils have been reported. (16).Vasudevan et al. (17) investigated the role of plants in the secretion of stomach acid and suggested that some ingredients may lead to maximum gastric acid secretion like chili while substances such as trachyspermum ammi may increase acid secretion by cholinergic mechanisms. Boskabady and colleagues (18) studies showed that the herb trachyspermum ammi has a calcium antagonists effect, leading to lower blood pressure, reduce spasms and has a bronchial dilation effect. Boskabadi studies on guinea pig's trachea showed a relaxant, an anti-histamine (H1) and an antitussive effect. Dr Rezvani and colleagues Studies (19) also showed that the aqueous extract of trachyspermum ammi can reduce seizures in laboratory animals. In addition, trachyspermum ammi anticonvulsant doses can have sedative effects. Other studies show (20) trachyspermum ammi extract is effective on chronic toxicity of bronchial epithelial cells and that the most of the active ingredient is volatile oil, which contains thymol.
Trachyspermum ammi herb is used in traditional medicine as a reliever of irritation, itching, neuropathic pain and headaches (21). Trachyspermum ammi fruit with the scientific name (Carum Copticum) is of the umbelliferous and its active substances is in the fruit, which contains thymol, that is stored in the secretory part (22). The analgesic effect of the plant extract on chronic pain suggests that trachyspermum ammi significantly reduces formalin-induced pain(23). The effect also is seen in the essential oils. Since the role of particular type of receptor involved in analgesic effects is inevitable, we are going to demonstrate the role of opioid, serotonergic and cholinergic receptors in analgesic effect of trachyspermum ammi using the formalin test.

Methods

A. Essences Preparation

For essential oils, herb seeds taken from the Agricultural Research Center of Yazd was used. This plant with the herbarium number 1-0303-293 has the approval of medicinal Plants Research Center of Yazd. In the Essences preparation, we add 500 ml of water in 100 grams of dried fruit, and then we have done water distillation with gentle heat(24). Then we attached the balloons to Clevenger apparatus and after heating, we gained 5 ml of essences and kept in the fridge until trial. Using saline solution with a concentration of 100 µl / kg, were injected intraperitoneally to animals in a volume of one ml.

B. Grouping the animals

For this study, 20 male white laboratory mice (Syrian) weighing 20-25 g in animals centre of medical School are selected randomly, and all under the same conditions of climate, nutrition and lighting were divided into 4 groups of five which one of them was the control group. Animals in the control group received a detemined concentration of Essences. The test groups also received a detemined concentration of Essences after injection of opioid, cholinergic and serotonergic inhibitors separately before formalin injection. After 10 minutes we have injected 20 ml of 2% formalin solution in animals feet subcutaneously.Animal response to this painful stimulus were recorded for an hour at intervals of 15 seconds.

C. Formalin Test

In the formalin test, we placed each animal in the experimental apparatus (including a glass table to keep and a mirror to see the movements of the animal). The solution with a concentration of 100 µl / kg (25) essence was given to control group. Naloxone at a dose of 5 mg/kg as an opioid receptor inhibitor, atropine 1 mg/kg as a cholinergic receptor inhibitor and ondansetron 0.5 mg/kg as a serotonergic receptor inhibitor, which reached one ml with normal saline, injected intraperitoneally in animals of test groupThe Results were recorded according to the method described by Dubuisson, D., and Dennis.SG (26).
In this survey every 15 seconds, based on animal behavior and pain in the formalin injected feet a

score of zero to three will be given (When the animal uses the injected foot like the other one, the score is zero. When he puts the injected on the ground but no relying on, the score is one. When he keeps the injected foot up, the score is two and if he shakes and bites the injected foot, the score is three). Then the pain intensity measured at intervals of 5 minutes and rates will be averaged and the average rate of pain in each animal is used for the statistical analysis. Subcutaneous injection in the formalin test causes pain in two steps. The first stage is rapid and severe pain that reaches maximum intensity within 5 minutes and in 10 minutes relieves the intensity. Again 20 minutes after the formalin injection, second phase of pain which is the chronic pain started and continues until 60 minutes after the test. Trachyspermum ammi extract, atropine, naloxone and ondansetron are used 15 minutes before of formalin injection.

D. Data Analysis

In each test period, mean pain intensity calculated for each animal for an hour and then the average of pain scores in every 5 minutes period was recorded. To compare the pain intensity in different groups, ANOVA and Tukey's tests was used and the difference ($p<0.05$) was considered as for significance.

Results

1. The analgesic effect of the essential oil of Trachyspermum ammi in the formalin test:

Our results show that the subcutaneous injection of formalin in the control animals' feet causes a pain in 2 phases which main intensity was in the first minute and then in the 20 minutes, we have recorded a decline in the intensity. The main intensity in second phase was for 25 minutes and then followed by a decline at the end of 35 minutes; the pain remained at the same level (Figure 1). Trachyspermum ammi oil injection also caused a biphasic curve which caused the maximum pain in 25 minutes (0.98±0.3) But in an hour, compared with the control group showed a significant decrease (0.19±0.0) ($p<0.05$). Severity of pain is based on the (26) Dubuisson, D., and Dennis.SG.

2. Mean pain intensity in the group that received naloxone initially and then Trachyspermum ammi essential oil:

Pain intensity did not show a significant decrease in the group that received 5 mg/kg naloxone initially, and then Trachyspermum ammi essential oil, which represents non-interference in the opioid receptors and analgesic effects of the essential (0.68±0.82) (figure 2)

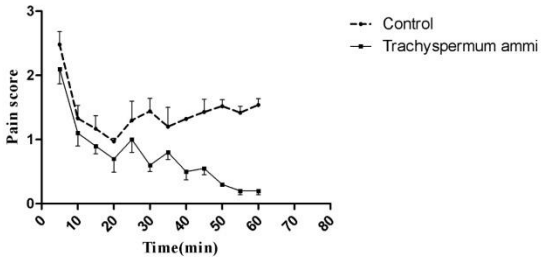

Figure 1. Mean pain intensity during formalin test (5-minute periods) in the control group and the group that received the essential oil of Trachyspermum ammi (2.18±0.2) and followed by pain relief until the twentieth minute. The main intensity in second phase was for 25 minutes (0.98±0.3) and then followed by a decline at the end of 35 minutes (n=5). * shows the significance ($p<0.05$)

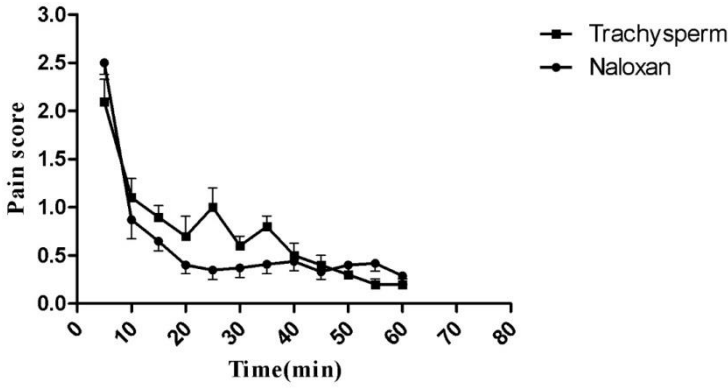

Figure 2. Comparison of mean pain intensity in the group that received naloxone initially and then Trachyspermum ammi essential oil (n=5). Pain intensity did not show a significant decrease, which represents non-interference in the opioid receptors and analgesic effects of the essential (0.68±0.82)

3. Mean pain intensity in the formalin test in the group that initially received ondansetron and then trachyspermum ammi essential oil:

Our evaluation on the severity of pain in the ondansetron group (0.5 mg/kg as a serotonergic receptor inhibitor) and then received trachyspermum ammi oil also does not show a significant decrease, which represents non-interference in the serotonergic receptors and analgesic effects of the essential oil (0.8 ±0.19) (Figure 3)

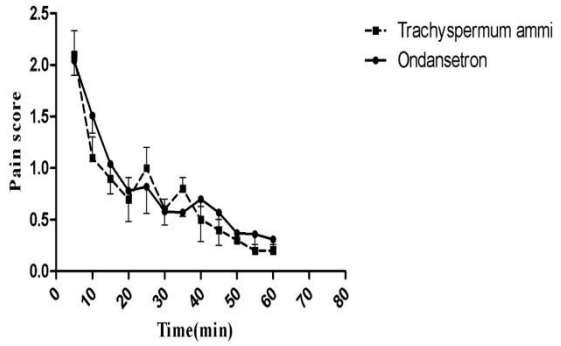

Figure 3. Comparison of mean pain intensity in the group that received ondansetron initially and then Trachyspermum ammi essential oil (n=5). Our evaluation on the severity of pain in the ondansetron group (0.5 mg/kg as a serotonergic receptor inhibitor) and then received trachyspermum ammi oil also does not show a significant decrease, which represents non-interference in the serotonergic receptors and analgesic effects of the essential (0.8 ±0.19)

4. Mean pain intensity in the formalin test in the group that initially received atropin and then trachyspermum ammi essential oil:
Our evaluation on the severity of pain in the atropin group (1 mg/kg as a cholinergic receptor inhibitor) and then received trachyspermum ammi oil showed a significant decrease, which represents interference in the cholinergic receptors and analgesic effects of the essential oil (1.77±0.54) (Figure 4)

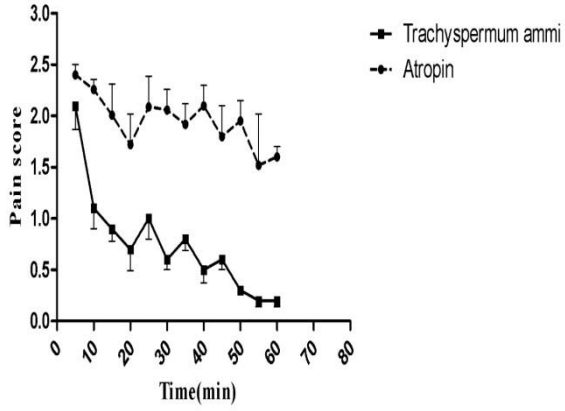

Figure 4. . Comparison of mean pain intensity in the group that received Atropin (1 mg/kg as a cholinergic receptor inhibitor) initially and then Trachyspermum ammi essential oil (n=5) shows a significant decrease, which represents interference in the CHOLINERGIC receptors and analgesic effects of the essential (1.77±0.54)

5. Mean pain intensity in the control group and the group that Received trachyspermum ammi oil, ondansetron, naloxone and atropine respectively:
The results of the one-hour average pain intensity during the formalin test shows no differences between the groups have received trachyspermum ammi oil, ondansetron and naloxone, respectively, while reduction in pain intensity was seen in the atropine and the essential oil of trachyspermum ammi group (P<0.05) (see Figure 5).

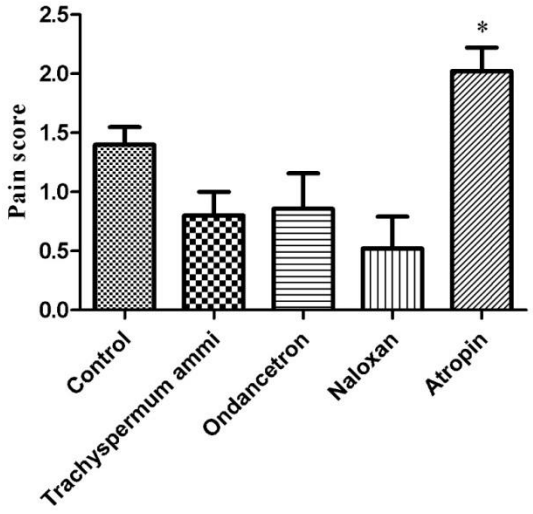

Figure 5. The results of the one-hour average pain intensity during the formalin test shows no differences between the groups have received trachyspermum ammi oil, ondansetron and naloxone, respectively, (n=5) while reduction in pain intensity was seen in the atropine and the essential oil of trachyspermum ammi group (P<0.05)

Discussion

Receptor-mediated analgesic effects of drugs are different. Among them will be noted, cholinergic receptor (27), receptors of nitric oxide (28) and non-opioid receptors (29). There is also material which are effective by non-opioid mechanisms, and through the cholinergic system (30). In physiological conditions, internal opioids are activated by interaction with the opioid receptors in the central nervous system in the spinal cord and higher centers and play a role in modulating pain. Antagonists of various materials inhibited this effect, most notably naltrexone and naloxone that are net opioid receptors and their effects exert by inhibition of adenylate cyclase and decreased cAMP.

Present study shows the inhibitory effect of naloxone, ondansetron and atropine to reduce the analgesic effect of the essential oil of Trachyspermum ammi and explains that the analgesic effect of Trachyspermum ammi is

inhibited by atropine from the tenth minute until the end of the 60 minutes. This result may confirm the role of acetylcholine in the internal analgesic response that compatible with other studies. Yamamoto and colleagues study showed nicotinic cholinergic receptor may relieves pain through the opioid system. Trachyspermum ammi oil causes the analgesic effect by activating cholinergic receptors, and stimulation of opioid receptors (31). Another study also confirms the role of cholinergic signaling on the analgesic effects of opioid (32). Administration of cholinesterase inhibitors, which have the ability to cross the blood - brain barrier, causes analgesic effects by activating opioid receptors. Cholinergic muscarinic receptors are one of the main areas of analgesic effects in the spinal cord. These receptors are located in the superficial dorsal horn. The analgesic effects begins by administrating of cholinergic agonists in these areas. The analgesic effects of Trachyspermum ammi oil is most likely through the cholinergic receptors in the spinal cord. These tests have been carried in laboratory animals, while cholinergic agonists has not been tested in humans But examples of the analgesic effect of cholinesterase inhibitors, such as neostigmine in reducing the acute and chronic pain in humans has been reported. Therefore, cholinergic agonists and cholinesterase inhibitors could be used in the future as opioid analgesics (33).

In another experiment, cholinergic and anticholinergic effects was done on analgesic effects the drugs such as morphine, codeine, fentanyl and pentazocine and showed that the injection of anticholinergic drugs such as atropine and scopolamine, increases the morphine-induced muscle rigidity, which indicates the fact that the analgesic systems will not be affected by anti-cholinergic and cholinergic systems. In another study with hot plate test confirmed the role of anticholinergics in the increase of analgesic effects of morphine and fentanyl. Morphine and other opioids are not the same because of their analgesic mechanism (34).

Cobra toxin is an alpha neurotoxin, has an analgesic effects that leads to activation of cholinergic system and intraperitoneally atropine injection of 10 mg/kg, completely eliminates the analgesic effect of the poison (35). In another study (36) analgesic effects of morphine was examined by using a hot plate and it was found that intrathecal injection of 10 µg of atropine, significantly reduces the analgesic effects of morphine. This finding explains that the activation of a cholinergic cycle in the mouse spinal cord activates opioid effects of morphine.

These studies indicates the fact that the cholinergic system mediates analgesic effects and the agonist and antagonist cholinergic drugs affect opioid analagesic effects which requires further investigation in this direction.

What can be obtained from the above data is indicative of the fact that the analgesic effect of Trachyspermum ammi essential oil applied through the cholinergic receptors because with antagonizing of cholinergic receptors using atropine, analgesic effect of essence disappears and the pain of this group of mice indicates this issue.

In this study, the role of opioid, serotonergic and cholinergic receptors in the analgesic effects of essential oil of Trachyspermum ammi was studied using formalin test. And showed that the inhibitors of cholinergic (atropine), leads to an increase in the formalin-induced pain in presence of the essential oil of trachyspermum ammi. so it represent that the main mediator in analgesic effects of the essential oil of Trachyspermum ammi may be cholinergic system.

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