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Analgesic effect of trans-anethole via downregulation of hypothalamic orexin and melanin-concentrating hormone gene expression in the rat

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Abstract

Background and Objective: The role of the central nervous system in pain control is prominent via the regulation of neuropeptides. Plant derivatives such as trans-anethole could be effective due to analgesic properties. The present study investigated the analgesic effect of trans-anethole via modulation of hypothalamic orexin and melanin-concentrating hormone (MCH) gene expression in rats.

Materials and Methods: Twenty male Wistar rats (180-200 g) grouped into four groups of five rats (n=5). To induce pain, 50 µl of formalin was injected into the hind paws of the animals. The intact and formalin control groups received saline. Formalin induced pain groups received 150 or 250 mg/kg of trans-anethole. Pain score was evaluated by performing the formalin test. The hypothalamic samples were removed to analyze the gene expression levels via real-time PCR technique.

Results: The pain score decreased in rats receiving 150 or 250 mg/kg trans-anethole compared to formalin control group. The relative gene expression of Orexin and MCH significantly increased in the formalin group compared to the intact rats. Injection of 150 or 250 mg/kg trans-anethole significantly reduced the relative gene expression of orexin and MCH in formalin induced pain groups compared to the formalin control rats.

Conclusion: Trans anethole caused downregulation of hypothalamic orexin and MCH gene expression due to its pain relieving properties. So, analgesic effects of trans anethole may be mediated via central mechanism.

Keywords: Trans-anethole, Melanin-concentrating hormone, Orexin, Pain

1. Introduction

Pain is an unpleasant feeling experience that is influenced by physiological, psychological and emotional processes (1). Pain classified into two types, acute and chronic. Acute pains are resolved after the removal of the harmful stimulus, but the pain may continue despite the removal of the stimulus and the apparent recovery of the body (2). The hypothalamus, especially the lateral hypothalamic area, is one of the most important areas of the central nervous system that plays a role in controlling pain and stress. The function of the lateral hypothalamus in regulating vital body functions has become an important research topic. Among these functions, the role of inflammatory pain regulation is important. Also, the lateral hypothalamus is a place for integrating autonomic and endocrine responses such as the pituitary gland and homeostatic balance, and it could receive and respond to peripheral pain stimuli (3,4).

In the hypothalamus, various neuropeptides are synthesized, each of them could play an important role in the regulating the body. Among them, orexin and melanin-concentrating hormone (MCH) are considered to be the most important ones. The MCH is synthesized as a neuromodulator by neurons located in the lateral hypothalamus and zona incerta. The MCHergic neurons distributed throughout the central

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nervous system such as the hypothalamus, somatosensory cortex, hippocampus, and amygdala, suggesting the involvement of MCH in different physiological functions (5-7). Also, it has been shown that MCH significantly increases mechanical and thermal pain thresholds (8). Orexin in the central nervous system is mainly synthesized in the lateral hypothalamus. In addition to the hypothalamus, this neuropeptide is also produced in peripheral organs such as adrenal glands, stomach, intestine, pancreas and testis (9). The role of the orexinergic system in various processes such as the reward system, energy homeostasis, sensory modulation, stress, cognition, endocrine functions and pain modulation has been proven (10-11).

Various drugs such as opioids are used to relieve chronic pain. However, these treatments often are limited due to their side effects (12). Many studies have shown that the treatment method using medicinal plants could control the level of pain. Plants such as fennel and anise contain an important compound called trans anethole (13). Studies show that the trans-anethole has antioxidant, anxiolytic, analgesic, anti-inflammatory and phytoestrogen properties (14). In this study, the analgesic effect of trans anethole was investigated via regulating the gene expression of orexin and MCH in formalin induced pain model rats.

2. Materials and Methods

2.1. Animal groups and treatment

For this study, trans-anethole was purchased from Sigma Aldrich (U.S.A). Twenty male Wistar rats were divided into four groups (n=5) and kept at laboratory conditions with free access to water and food. The laboratory temperature was set at 23-25°C under 12 h dark/light cycle. The intact and formalin control groups received saline only. Two formalin induced pain groups received single dose of 150 or 250 mg/kg trans-anethole intraperitoneally. All injections were made at 9-11 a.m. (15, 16).

2.2. Formalin-induced pain

In order to induce pain, 50 µl of 5% formalin injected subcutaneously into the hind paw using 30-gauge syringe. The animal placed in a box 30 x 30 x 30 cm with an adjusted mirror under it (45 angle). Immediately, pain behaviors evaluated for 60 min. The pain score calculated according to the following formula for each group. Zero behavior: when the animal places both paws comfortably on the floor, behavior 1: when the animal keeps the tip of the injected paw on the floor, behavior 2: When the animal holds the injected paw above the body, behavior 3: when the animal licks the injected paw. Pain score in 5 minutes = (0T0+1T1+2T2+3T3)/300. T1, T2, T3, T0 time period (seconds) when the animal shows zero, one, two and three behaviors (17,18).

2.3. Real time PCR

Hypothalamus samples homogenized in 1 ml Pure Zol (Bio Rad Co., US). The homogenized solution placed on ice for 5 min, then 200 µl chloroform added to the homogenized solution. The solution shaken for 15 sec and centrifuged for 15 min at 12,000 g and temperature of 4 Co. Then, 500 µl isopropanol added to the separated surface solution. The resulting solution shaken for 15 sec and placed on ice for 5 min. Then solution centrifuged for 10 min at 12000 g and 4 Co. The obtained white precipitate is the total RNA. RNA concentration determined using a nanodrop device. Then 1µg of RNA used for cDNA synthesis according to the kit protocol (Thermo Scientific Co., U.S.A). 1 µg RNA, 1 µg primer oligothymine (40 □M) and 1 µg nucleotide triphosphates mixture (10 mM dNTP) transferred to a microtube (0.2 ml). The volume of the solution reached 10 µl by adding nuclease free water. The resulting solution placed in a thermal cycler at a temperature of 65oC for 5 min. In the next step, 1 µl (unit 100) of M-MuLV enzyme, 2 µl buffer X M-MuLV10, 1 µl RNase inhibitor and 6 µl nuclease free water added to 10 µl of the previous solution so that the reaction volume reached 20 µl. The resulting solution was incubated in a thermal cycler. SYBR Green I kit (Takara Bio Inc., Japan) and especial primers) orexin, MCH and GAPDH (were used for amplification by Real Time PCR technique. The samples were amplified in the PCR system according to the following program: One cycle (2 min, 95°C) and 40 cycles (95°C for 5 s, 60°C for 20 s, and 60°C for 25 s). Nucleotide sequences for sense and antisense primers mentioned below were used to amplify the genes. Orexin, F: 5-CTCCTTCAGGCCAACGGTAA -3; R: 5-AGGGCAGGGATATGGCTCTA-3; MCH: 5-TCAGAAGGAAGATACCGCAGA-3, R: 5-ACTGCTGGTCCT TTCAGAGC-3; GAPDH: F: 5-AAGTTCAACGGCACAGTCAAG -3, R: 5-CATACTCAGCACCAGCATCAC-3. The relative gene expression was calculated using equation $^{2-\Delta\Delta CT}$ (19)

2.4. Statistical analysis

The collected data were analyzed using SPSS version 23 using one-way ANOVA and Post hoc Tukey test. The results were presented as mean ± standard deviation of means (± SEM). Significance was defined by $P < 0.05$.

3. Results

As shown in Figure 1, the pain score significantly decreased in rats receiving 150 mg/kg of trans-anethole in the time period of 5, 20, 25, 30 min compared to the formalin control group ($P < 0.05$). Also, a significant decrease in the pain score was observed in rats receiving 250 mg/kg trans-anethole in the time period of 5, 20, 25, 30, 40 and 50 min

compared to the formalin control group (P <0.05). The pain score in phase 1 (0-5 min) and phase 2 (15-60 min) in the group receiving trans-anethole either 150 or 250 mg/kg significantly reduced compared to the formalin control group (P <0.05) (Figure 2).

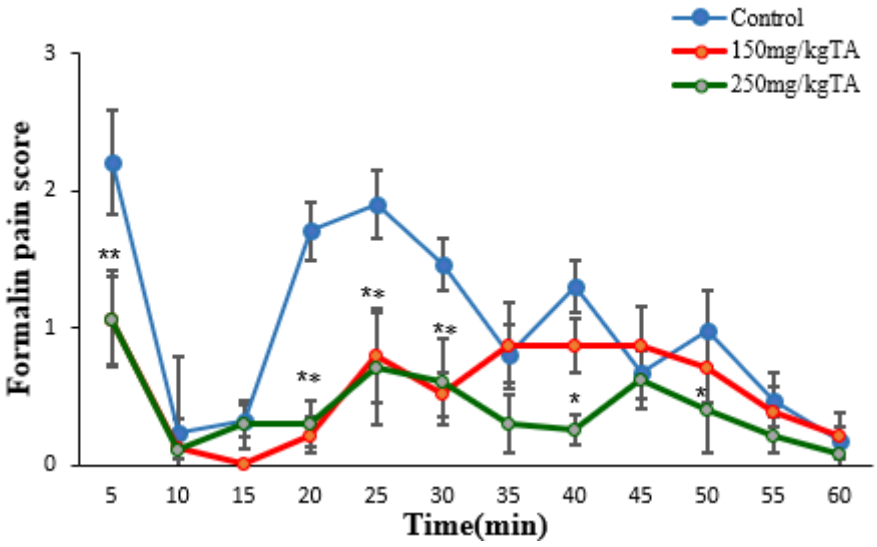

Figure 1. The effect of trans anethole on score pain in formalin model rats (0-60 min). The results are presented as mean± standard error of mean (SEM) and significance was defined by P<0.05.

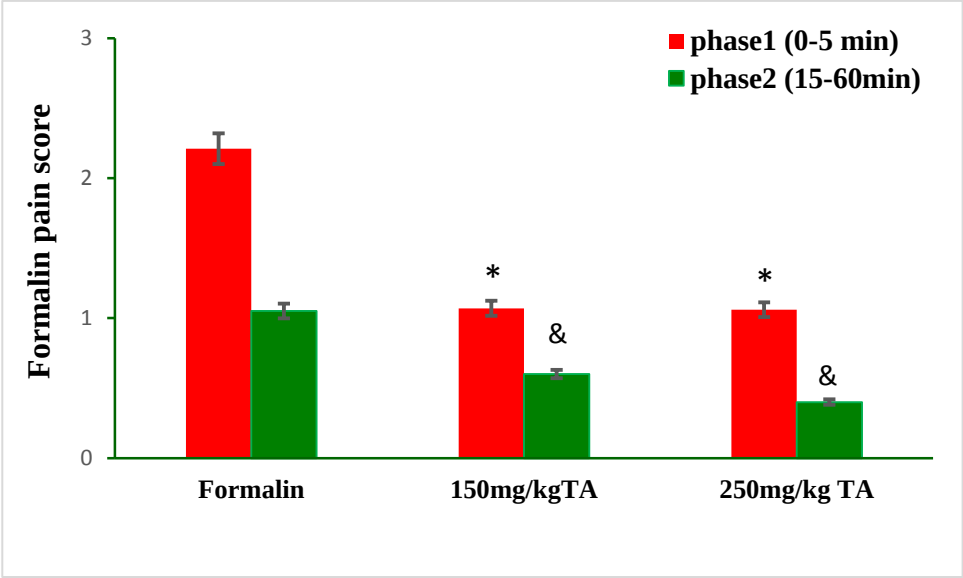

Figure 2. The effect of trans anethole on score pain in formalin model rats (phase 1 and phase 2). The results are presented as mean± standard error of mean (SEM) and significance was defined by P<0.05. *: compared to formalin group phase 1; &: compared to formalin group phase 2

The mRNA level of orexin in the formalin group significantly increased compared to the intact group receiving normal saline (P <0.05). Also, the mRNA level of orexin in the formalin group receiving trans-anethole at either 150 mg/kg or 250 mg/kg significantly decreased compared to the formalin group receiving normal saline (P <0.05) (Figure 3).

As shown in Figure 4, the mRNA level of MCH significantly increased in the formalin group compared to the intact group receiving normal saline. Also, injection of trans-anethole at either 150 mg/kg or 250 mg/kg caused a significant decrease in the mRNA level of MCH in the formalin group compared to the intact group receiving normal saline (P <0.05).

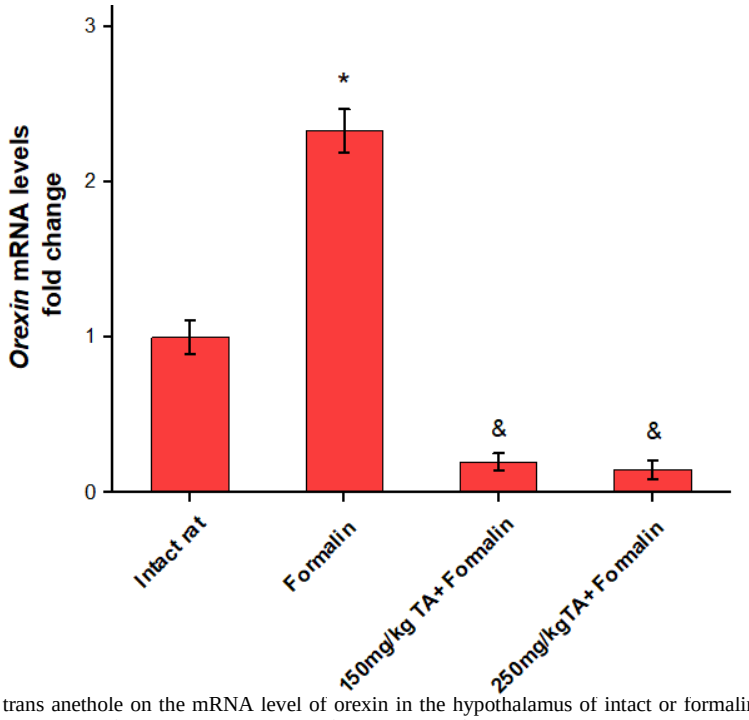

Figure 3: The effect of trans anethole on the mRNA level of orexin in the hypothalamus of intact or formalin model rats. The results are presented as mean± standard error of mean (SEM) and significance was defined by P<0.05. *: compared to intact rats; &: compared to formalin control group.

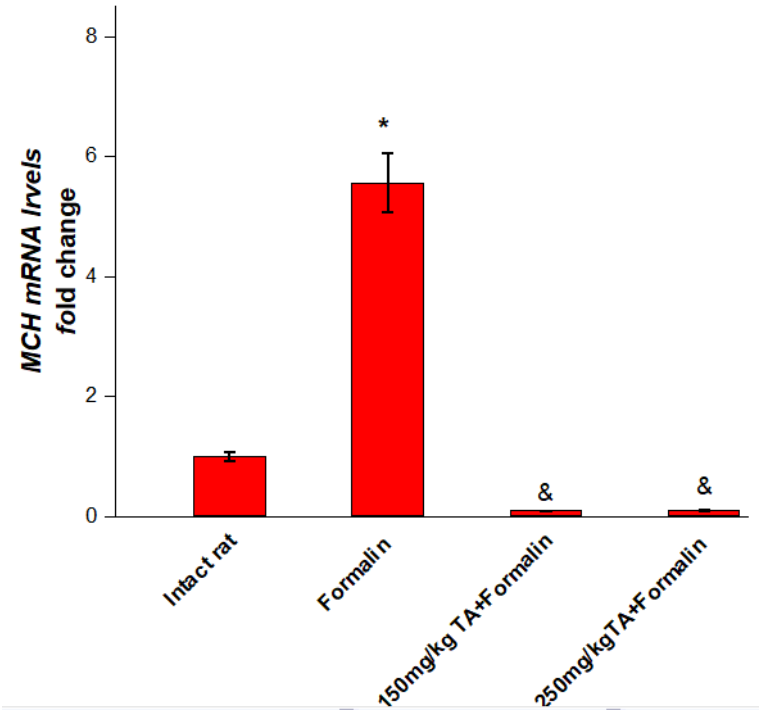

Figure 4: The effect of trans anethole on the mRNA level of MCH in the hypothalamus of intact or formalin model rats. The results are presented as mean± standard error of mean (SEM) and significance defined by P<0.05. *: compared to intact; &: compared to formalin group.

4. Discussion

In the present study, behavioral and molecular

approaches were used in order to investigate the analgesic effects of trans-anethole in rats. The pain score in the rat's model of pain showed an increase compared to the control. The results were consistent with many previous studies that reported pain behaviors induced by formalin injection (20). Considering that the use of chemical or opioid drugs in pain control may cause complications and disorders in the function of neuroendocrine axes, including reproduction and the hypothalamus-pituitary-adrenal axis (21,22). In recent years, various researches have been conducted in find of drugs painkillers from derivatives to minimize the side effects of chemical drugs in patients with chronic pain. Also, we found that the administration of trans-anethole reduces pain intensity via regulating hypothalamic genes. The present results were in accordance with the previous researches which showed the analgesic effects of trans-anethole in different pain models such as the acetic acid -induced pain, the glutamate -induced pain and the formalin -induced pain (15,22).

Trans-anethole is one of the most effective compounds derived from medicinal plants such as fennel (Foeniculum vulgare) and anise (Pimpinella anisum). Previous studies showed that anise oil, like morphine and aspirin, has analgesic effects in pain model rats (23). Consumption of methanol extract of fennel fruit exerts analgesic and anti-inflammatory effects (24). Amota et al in 2014 showed that intraperitoneal injection of fennel extract to rats inhibited acetic acid -induced pain and formalin -induced pain (25). Although previous researches have confirmed the analgesic effects of anise, fennel and their effective compound, trans-anethole, by behavioral tests, but no research has been done on the molecular mechanisms of trans-anethole in reducing pain.

Different areas of the central nervous system, including the spinal cord, brain stem, hypothalamus, hippocampus, etc., involved in understanding, processing and relieving pain. The lateral hypothalamus region is one of the most important regions involved in creating the phenomenon of analgesia. In addition, several neuropeptides and neurotransmitters have role in the analgesia, including two neuropeptides in the lateral hypothalamus, such as Orexin and melanin-concentrating hormone (MCH) (26).

Orexin neurons are mainly concentrated in the lateral hypothalamus. In addition to controlling wakefulness, food intake, reproduction, orexin plays an important role in controlling inflammatory pain (27). Lateral hypothalamus stimulation causes analgesic effects in inflammatory pain models (28). Also, studies show that these areas receive multiple pain inputs from the spinal-thalamic pathway. In addition to pain control, the lateral hypothalamus is important in stress regulation through functional communication with the hypothalamic-pituitary-adrenal (HPA) axis (29, 30). Trans-anethole has anti-inflammatory and antioxidant activity and it can play a role in reducing HPA axis activity by reducing inflammatory factor and free radicals (31). One of the factors that stimulate the HPA axis is the increase in CRH activity. Evidence suggests that cortisol increases CRH levels (32). Also, there is a close relationship between increased CRH and pain (33). Therefore, trans-anethole by reducing the inflammatory factor and oxidative stress, leads to a decrease in the expression level of cortisol and CRH, which can reduce the level of pain.

MCH is a 19 amino acid neuropeptide and also it is important in the regulation of nutrition and energy homeostasis (34). In addition, these studies show that the MCH system plays a role in regulating emotions, stress responses and increases the activity of the hypothalamus-pituitary-adrenal axis. The present research showed that the expression of MCH was high in pain model rat. We also observed that trans-anethole reduces the level of MCH in pain model rats. Because trans-anethole has estrogenic properties (35). Estrogen also reduces pain (36). Therefore, it is possible that trans-anethole reduces MCH gene expression through its estrogenic effects, leading to pain relief. Therefore, to obtain more accurate evidence of the real relationship between MCH, pain and trans-anethole, such studies lead to the recognition of useful drugs for pain relief.

Conclusion

Trans-anethole improves the pain score in the pain model rats. Pain relieving properties of trans- anethole may be involved in downregulation of hypothalamic orexin and MCH gene expression in formalin- induced pain model rats. Therefore, trans-anethole may be suggested as a therapeutic option for pain relief via central mechanisms of pain management.

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Ethical Considerations

Compliance with ethical standards

All the testing process was carried out under the supervision of the Ethics Committee of Mohaghegh Ardabili University (code: IR.UMA.REC. 1400.038).

Conflict of interest

The authors declared no conflict of interest

References

1. Alshami AM. Pain: Is it all in the brain or the heart?. Current Pain and Headache Reports 2019; 23:1-4.
2. Lavand'homme P. The progression from acute to chronic pain. Current Opinion in Anesthesiology 2011;24(5):545-50.
3. Ezzatpanah S, Babapour V, Sadeghi B, Haghparast A. Chemical stimulation of the lateral hypothalamus by carbachol attenuated the formalin-induced pain behaviors in rats. Pharmacology Biochemistry and Behavior 2015; 129:105-10.
4. Wang D, Pan X, Zhou Y, Wu Z, Ren K, Liu H, Huang C, Yu Y, He T, Zhang X, Yang L. Lateral septum-lateral hypothalamus circuit dysfunction in comorbid pain and anxiety. Molecular Psychiatry 2023; 16:1-1.
5. Jang JH, Park JY, Oh JY, Bae SJ, Jang H, Jeon S, Kim J, Park HJ. Novel analgesic effects of melanin-concentrating hormone on persistent neuropathic and inflammatory pain in mice. Scientific Reports 2018; 8(1):707.
6. Lin Y, Quartermain D, Dunn AJ, Weinshenker D, Stone EA. Possible dopaminergic stimulation of locus coeruleus α1- adrenoceptors involved in behavioral activation. Synapse 2008; 62(7):516-23.
7. Hooten WM. Chronic pain and mental health disorders: shared neural mechanisms, epidemiology, and treatment. InMayo Clinic Proceedings 2016; 91(7):955-970.
8. Mahboubi M. Foeniculum vulgare as valuable plant in management of women's health. Journal of menopausal medicine 2019;25(1):1-4.
9. Martins, I. et al. Noradrenaline increases pain facilitation from the brain during inflammatory pain. Neuropharmacology 2013; 71: 299–307.
10. Memon T, Yarishkin O, Reilly CA, Križaj D, Olivera BM, Teichert RW. trans-Anethole of fennel oil is a selective and nonelectrophilic agonist of the TRPA1 ion channel. Molecular Pharmacology 2019; 95(4):433-41.
11. Mesfin M, Asres K, Shibeshi W. Evaluation of anxiolytic activity of the essential oil of the aerial part of Foeniculum vulgare Miller in mice. BMC complementary and alternative medicine 2014;14(1):1-7
12. Borgland SL, Taha SA, Sarti F, Fields HL, Bonci A. Orexin A in the VTA is critical for the induction of synaptic plasticity and behavioral sensitization to cocaine. Neuron 2006;49(4):589-601.
13. Boutrel B, Kenny PJ, Specio SE, Martin-Fardon R, Markou A, Koob GF, de Lecea L. Role for hypocretin in mediating stress-induced reinstatement of cocaine-seeking behavior. Proceedings of the National Academy of Sciences 2005;102(52):19168-73.
14. Camurça-Vasconcelos AL, Bevilaqua CM, Morais SM, Maciel MV, Costa CT, Macedo IT, Oliveira LM, Braga RR, Silva RA, Vieira LS. Anthelmintic activity of Croton zehntneri and Lippia sidoides essential oils. Veterinary Parasitology 2007 ;148(3-4):288-94.
15. Ritter AM, Ames FQ, Otani F, de Oliveira RM, Cuman RK, Bersani-Amado CA. Effects of anethole in nociception experimental models. Evidence-Based Complementary and Alternative Medicine 2014 ; 1-7..
16. Vastegani SM, Khoshnam SE, Mansouri E, Hajipour S, Ghafouri S, Bakhtiari N, Sarkaki A, Farbood Y. Neuroprotective effect of anethole against rotenone induced non-motor deficits and oxidative stress in rat model of Parkinson's disease. Behavioural Brain Research 2023; 437:100-114.
17. Melo Júnior JD, Damasceno MD, Santos SA, Barbosa TM, Araújo JR, Vieira-Neto AE, Wong DV, Lima-Júnior RC, Campos AR. Acute and neuropathic orofacial antinociceptive effect of eucalyptol. Inflammopharmacology 2017; 25:247-54.
18. Radmehr M, Jahromi HK, Abedi H, Seifi V, Karami S, Jahromi ZK. Comparison of the effect of lycopene with ibuprofen on sensory threshold of pain using formalin test in adult male rats. Journal of Chemical and Pharmaceutical Research 2016; 8(4):1322-7.
19. Asadian M, Yaghoubi H, Mahmoudi F, Gollo KH. Effect of Trans-Anethole on Gene Expression of Steroidogenic Enzymes in the Ovary of Polycystic Ovary Syndrome Model Rate. Galen Medical Journal 2022;11: e2219.
20. Meymandi MS, Sepehri G, Izadi G, Zamiri Z. Evidence for antinociceptive effects of combined administration of vitamin E and celecoxib in tail-flick and formalin test in male rats. Pharmacological Reports 2019; 71(3):457-64.
21. Fountas A, Van Uum S, Karavitaki N. Opioid-induced endocrinopathies. The Lancet Diabetes & Endocrinology 2020; 8(1):68-80.
22. Wang B, Zhang G, Yang M, Liu N, Li YX, Ma H, Ma L, Sun T, Tan H, Yu J. Neuroprotective effect of anethole against neuropathic pain induced by chronic constriction injury of the sciatic nerve in mice. Neurochemical research 2018; 43:2404-22.

23. Tas A, Ozbek H, ATASOY N, Altug M, CEYLAN E. Evaluation of analgesic and anti-inflammatory activity of Pimpinella anisum fixed oil extract. Indian Veterinary Journal 2006;83(8).

24. Choi EM, Hwang JK. Antiinflammatory, analgesic and antioxidant activities of the fruit of Foeniculum vulgare. Fitoterapia 2004; 75(6):557-65.

25. Elizabeth AA, Josephine G, Muthiah NS, Muniappan M. Evaluation of analgesic and anti-inflammatory effect of Foeniculum vulgare. Res. J. Pharm. Biol. Chem. Sci 2014; 5:658-68.

26. Fakhoury M, Salman I, Najjar W, Merhej G, Lawand N. The lateral hypothalamus: an uncharted territory for processing peripheral neurogenic inflammation. Frontiers in neuroscience 2020; 14:101.

27. Zhou W, Cheung K, Kyu S, Wang L, Guan Z, Kurien PA, Bickler PE, Jan LY. Activation of orexin system facilitates anesthesia emergence and pain control. Proceedings of the National Academy of Sciences. 2018;115(45): E10740-7.

28. Jeong Y, Wagner MA, Ploutz-Snyder RJ, Holden JE. Pain condition and sex differences in the descending noradrenergic system following lateral hypothalamic stimulation. IBRO reports 2020; 8:11-7.

29. Jahangirvand M, Yazdi F, Moradi M, Haghparast A. Intra-accumbal orexin-1 receptors are involved in antinociception induced by stimulation of the lateral hypothalamus in the formalin test as an animal model of persistent inflammatory pain. Iranian Journal of Pharmaceutical Research: IJPR 2016;15(4):851.

30. Bomholt SF, Harbuz MS, Blackburn-Munro G, Blackburn-Munro RE. Involvement and role of the hypothalamo-pituitary-adrenal (HPA) stress axis in animal models of chronic pain and inflammation. Stress 2004;7(1):1-4.

31. Kim KY, Lee HS, Seol GH. Anti-inflammatory effects of trans-anethole in a mouse model of chronic obstructive pulmonary disease. Biomedicine & Pharmacotherapy 2017; 91:925-30.

32. Vgontzas AN, Fernandez- Mendoza J, Lenker KP, Basta M, Bixler EO, Chrousos GP. Hypothalamic–pituitary–adrenal (HPA) axis response to exogenous corticotropin- releasing hormone (CRH) is attenuated in men with chronic insomnia. Journal of Sleep Research 2022;31(3): e13526.

33. Murala S, Nagarajan E, Bollu PC. Galanin, Substance P, and Melanin-Concentrating Hormone. In Neurochemistry in Clinical Practice 2022; 255-263.

34. He X, Li Y, Zhang N, Huang J, Ming X, Guo R, Hu Y, Ji P, Guo F. Melanin-concentrating hormone promotes anxiety and intestinal dysfunction via basolateral amygdala in mice. Frontiers in Pharmacology 2022;13.

35. Negahdari FM, Gholamnezhad Z, Noshahr ZS, Keshavarzi Z. A comparison between the effect of trans-anethole and metformin on biochemical parameters of polycystic ovary syndrome in rats. Avicenna Journal of Phytomedicine 2021;11(5):484.

36. Zhang W, Wu H, Xu Q, Chen S, Sun L, Jiao C, Wang L, Fu F, Feng Y, Qian X, Chen X. Estrogen modulation of pain perception with a novel 17β-estradiol pretreatment regime in ovariectomized rats. Biology of sex Differences 2020; 11:1-3.