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Original Article:

The Effects of Trans-anethole on the Hypothalamic *CGRP* and *CRH* Gene Expression in Rat Model of Stress

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Abstract

Introduction: Stress is defined as a physiological response to environmental conditions which could cause changes in the level of neuropeptides in the central nervous system. Trans- anethole is the secondary active compound with anti-stress and antioxidant properties. This research investigates the effects of trans-anethole on the hypothalamic *CRH* and *CGRP* gene expression in stress model rats.

Materials and Methods: Twenty male rats weighing 200-220 g were used. Animals were divided into four groups (n=5). The intact control or stress groups received saline. Two stress groups received trans-anethole (150 mg/kg or 250 mg/kg, IP). Thirty minutes following the injection of drugs, animals were subjected to acute immobilization stress for two hours. Then, behavioral tests were performed. The hypothalamic samples were removed. *CRH* and *CGRP* gene expression was measured using RT-PCR.

Results: The mRNA levels of *CGRP* and *CRH* significantly increased in the stress group compared to those of the control. In rats receiving 150 mg/kg or 250 mg/kg of trans-anethole, the mRNA level of *CGRP* and *CRH* decreased significantly compared to that of the stress group. Also, injection of 150 mg/kg or 250 mg/kg of trans-anethole significantly improved the stressful behaviors compared to what happened in the stress group.

Conclusion: Trans-anethole may be considered as a potential anti-stress factor due to its inhibitory effects on the activity of hypothalamic stress pathways such as CRH and CGRP.

Keywords: CRH, CGRP, Stress, Trans-anethole.

1. Introduction

Stress is a physiological and psychological response to stress factors. When a person experiences stress, the body system responds to it with several mechanisms to maintain homeostatic conditions. The brain together with the peripheral system can play a role in controlling stress. Under conditions of stress, the changes that occur in the central nervous system include the release of corticotropin-releasing hormone (CRH) from the hypothalamus, which causes the release of Adrenocorticotropic hormone (ACTH) into the blood circulation. This hormone finally affects the adrenal glands and increases the release of cortisol, which is the stress hormone. An increase in cortisol can change the expression of different neuropeptides such as CRH and CGRP. These neuropeptides have physiological activities such as the control of the reproductive axis, stress and pain as well as the regulation of digestive and respiratory systems [1-3].

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide that belongs to the calcitonin (CT) family [4]. In humans and mice, CGRP is expressed in the testis, ovary, spleen, prostate, intestine, lung, heart, and kidney [5]. Studies have indicated that exposing humans or rodents to stressful factors increases CGRP [6]. Corticotropin-releasing hormone (CRH) in mammals consists of a 41 amino acid peptide. CRH is synthesized in neurons located in the hypothalamus and stimulates the release of ACTH (a 39 amino acid peptide) from the pituitary gland [7]. CRH neurons in the paraventricular nucleus of the hypothalamus are activated in response to stress. Studies have shown that disorder in the CRH system of the brain is related to depression and anxiety. Stress, by affecting CRH neurons, increases the activity and release of CRH. Finally, it causes the release of the stress hormone cortisol in the blood [8].

Trans-anethole, with the scientific name 'trans-1-methoxy4(-1-propenyl) benzene', is a basic combination of plants such as anise and fennel. Trans-anethole is an aromatic compound with many uses in food, pharmaceutical and perfume industries. Studies have proven that trans-anethole is a very hydrophobic compound and its formula is similar to that of the catecholamines (dopamine, epinephrine, and norepinephrine). Trans-anethole has strong steroidogenic and anti-inflammatory effects [9-12]. Unique analgesic, neuroprotective and anxiolytic therapeutic properties of trans-anethole have already been established, yet there is not, to the best of our knowledge, any information about molecular mechanisms underlying its anxiolytic effect. The present study aimed to investigate intra hypothalamic molecular mechanism by which trans-anethole may suppress stress-induced behaviors.

2. Materials and Methods

Chemicals and Animals

Trans-anethole was purchased from Sigma-Aldrich (USA). Twenty male Wistar rats weighing 200-220 g were prepared. For acclimatization, the rats were kept for two weeks in laboratory conditions with enough water, food and suitable temperature. The temperature was set at 22 ± 2 °C. The animals were under a photoperiod of 12 light/12 h dark. This experiment was approved by the ethics committee (code: IR.UMA.REC.1401.061).

Groups and experimental design

Rats were divided into groups of four (n=5). The drug was injected according to what follows: control and stress groups received saline; two groups under stress were injected with trans-anethole 150 mg/kg or 250 mg/kg (IP, single dose). All injections were performed 30 minutes before stress induction. The injection time was 9-10 a.m.

Induction of acute immobility stress (AIS)

Thirty minutes after the rats received the drug, the animals were placed in a 5 x 20 cm restraint cage for two hours. The animals were settled down in restraint cage in dark environment for two hours. Then, they were removed from restraint cage and ten minutes later behavioral tests were performed. After two hours of exposure to stress the rats were removed from the restraint and transfered to the cage ten minute later behavioral test were performed should be deleted.[13].

Behavioral tests

Open field test (OFT): This test is taken to evaluate stress in the animal. The open field box is 60 x 60 x 60 cm and its floor is divided into 16 equal squares. The four middle squares are considered as the center. To start the test, rats were placed in the center of the box. Then, the animal behavior was recorded with a camera for 5 minutes. The parameters measured by this test included the length of time spent in the center of the box, and the number of times entering the center [14].

Forced swimming test (FST): To perform the test, rats were first placed in a container (30 cm length, 30 cm wide, and 50 cm high) filled with two-thirds of water. The water temperature was set between 23-25^{o}C. The animal was allowed to swim freely in the water. The behavior was recorded by the camera for 6 minutes. Swimming, by definition, refers to the active movement of the arms and legs. Immobility is the stopping of the arms and legs. An increase in immobility time is considered equivalent to stress [15].

Sample collection

After the behavioral test, rats were anesthetized with ketamine-xylazine. Then, the heads of the animals were separated and the brains were removed. The ventral surface of the brain was placed upwards and a section with a thickness of 4 mm containing the hypothalamus was prepared. Immediately hypothalamus samples were kept at -80 temperature until RNA extraction. The mean relative expression of *CRH* and *CGRP* genes was measured using RT-PCR technique.

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA of all hypothalamic samples was extracted with TRIZol kit (Qiagen Co, Germany). Then, the concentration of RNA was determined with nanodrops (Thermo Fisher Scientific, Waltham, MA, USA) to read absorption at wavelengths of 260 and 280 nm. For cDNA synthesis, 1 µg of RNA total, and oligo-thymine primers were used according to the kit instructions. In order to measure relative gene expression levels, 1 µg of the synthesized cDNA was entered into the Real Time PCR reaction, and performed with the SYBR Green I kit (Takara Bio Inc., Japan). One cycle (15 min, 95 °C) and 40 cycles (95 °C for 20 s, 60 °C for 15 s, and 72 °C for 10 s) were considered for the PCR system. Nucleotide sequences for sense and antisense primers for *GAPDH*, *CRH* and *CGRP* genes were as what follows: *CRH*: F: 5′ TGGATCTCACCTTCCACCTTCTG -3′, R: 5′-CCGATAATCTCCATCAGTTTCCTG-3′, *CGRP*: F:5′ TCTAAGCGGTGTGGGAATCT--3′, R:5′-TAGGGGTGGTGGTTTGTCTC -3′, *GAPDH:* F: 5′ AAGTTCAACGGCACAGTCAAG -3′, R: 5′-CATACTCAGCACCAGCATCAC -3′. [16]. *GAPDH* was used as a housekeeping gene. The amount of gene expression changes was calculated by equation $2^{-\Delta\Delta CT}$.

Statistical analysis

The resulting data were analyzed using SPSS software (version 23), one-way ANOVA and Tukey's post hoc test. The results were presented as mean ± SEM. Values with P ≤0.05 were reported as significant.

3. Results

Anti-stress effects of trans-anethole on OFT and FST results

As shown in Fig 1, the length of time spent in the center decreased in the stress group compared to that of the control group. The decrease was significant (P ≤ 0.05). Also, the time spent in the center showed a significant increase in rats receiving 150 mg/kg or 250 mg/kg trans-anethole compared to those in the stress group (P ≤ 0.05).

In the stress group compared to the control group, a decrease in the number of entries into the center was observed, which was statistically significant (P ≤ 0.05). Also, the injection of 150 mg/kg and 250 mg/kg of trans-anethole caused a significant increase in the number of entering the center compared to that of the stress group (Fig 2) (P≤0.05).

The results of FST test showed that the duration of immobility increased significantly in the stress group compared to the control group (P ≤ 0.05). The duration of immobility in rats receiving 150 mg/kg and 250 mg/kg of trans-anethole decreased compared to that of the stress group, which was statistically significant only in the group receiving 250 mg/kg of trans-anethole (P ≤ 0.05) (Fig 3).

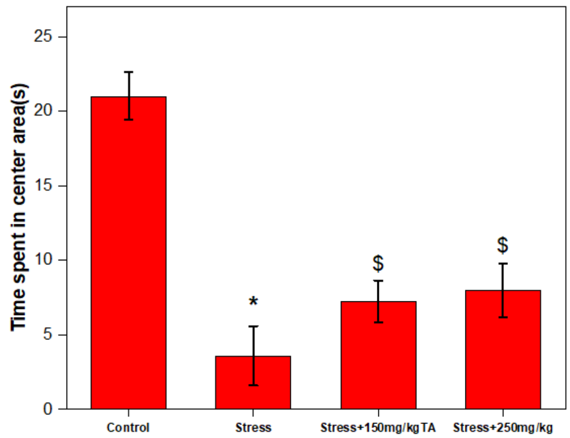

Fig 1. Effect of 150 mg/kg or 250 mg/kg Trans anethole (TA) on time spent center area in OFT. Data are shown as mean ± S.E.M, (p < 0.05) *: compared with control, $: compared with stress group

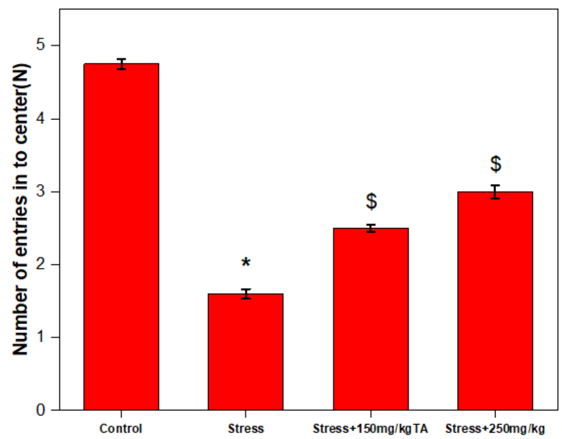

Fig 2. Effect of 150 mg/kg or 250 mg/kg Trans anethole (TA) on number of entries center in OFT. Data are shown as mean ± S.E.M, (p < 0.05) *: compared with control, $: compared with stress group

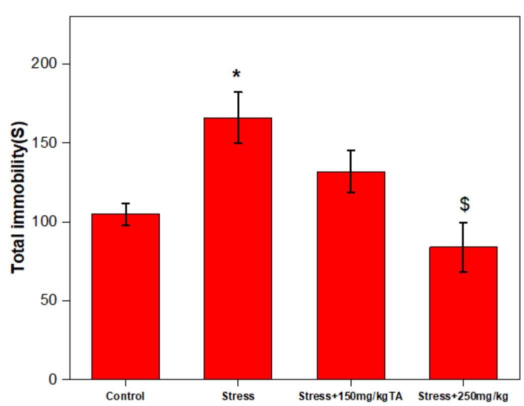

Fig 3. Effect of 150 mg/kg or 250 mg/kg Trans anethole (TA) on immobility time in FST. Data are shown as mean ± S.E.M, (p < 0.05) *: compared with control, $: compared with stress group

Effect of trans-anethole on the mRNA level of CRH and CGRP

The mRNA level of *CRH* in the stress group increased significantly compared to that of the control group (P ≤ 0.05). Also, in the group receiving 150 mg/kg or 250 mg/kg of trans anethole, the mRNA level of *CRH* decreased compared to that of the stress group. The decrease was statistically significant (P ≤ 0.05). (Fig 4).

As shown in Fig 5, the mRNA level of *CGRP* increased in the stress group compared to that of the control. The increase was significant (P ≤ 0.05). The mRNA level of *CGRP* in the group receiving 150 mg/kg or 250 mg/kg of trans anethole decreased significantly compared to that of the stress group.

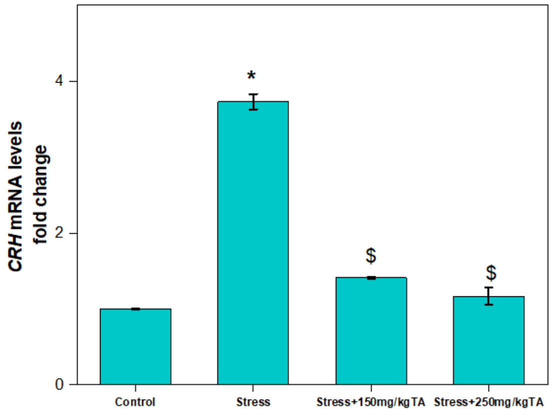

Fig 4. Effect of 150 mg/kg or 250 mg/kg Trans anethole (TA) on the mRNA level of *CRH*; Data are shown as mean ± S.E.M, (p < 0.05). *: compared with control, $: compared with stress group

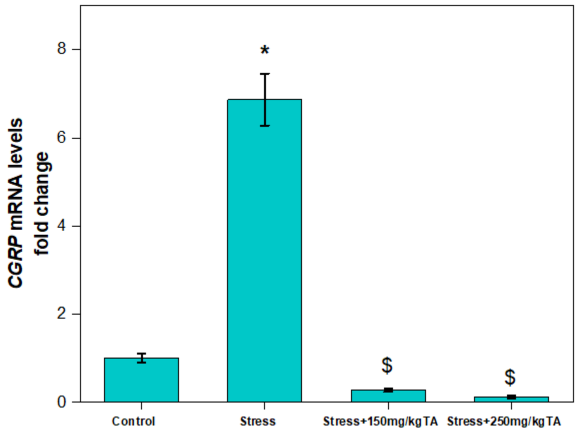

Fig 5. Effect of 150 mg/kg or 250 mg/kg Trans anethole (TA) on the mRNA level of *CGRP*; Data are shown as mean ± S.E.M, (p < 0.05). *: compared with control, $: compared with stress group

4. Discussion

The present study showed that rats receiving 150 mg/kg and 250 mg/kg of trans-anethole changed their stressful behaviors. Trans-anethole injection decreased the duration of immobility (swim test) (Fig 3). In addition, trans-anethole injection into rats increased the time spent in the center and the number of entries into the center (open field test) (Fig 1, 2). Besides, the injection of 150 mg/kg and 250 mg/kg of trans anethole decreased stress in rats compared to the control group (Figures 4, 5). This finding is in accordance with the research that reported trans anethole reduces stress [17]. Also, this study showed that stress increases the average relative expression of corticotropin-releasing hormone (CRH) genes. This finding is consistent with previous results [18, 19]. The average gene expression of calcitonin gene related peptide (CGRP) increased in the stress group compared to the control group. It has been reported in previous studies that inhibition of CGRP receptor signaling plays an important role in modulating anxiety [20]. In rats receiving 150 mg/kg and 250 mg/kg trans-anethole, the mean expression CGRP and CRH genes decreased.

Stress is a psychiatric disorder that is often accompanied by symptoms such as headache, sweating, palpitations, chest tightness, and stomach upset [21]. It can affect the physiological balance of the body, leading to the activation of a coordinated physiological response in the peripheral system and the central nervous system. Under the conditions of acute and chronic stress, areas of the brain can change, which causes changes in brain function. During acute stress, an immediate response leads to the activation of the sympathetic nervous system and the release of hormones that help the body cope with the stressor. Also, the activation of the stress system can lead to neuroendocrine, metabolic, and autonomic nervous system changes [22, 23].

Several studies have reported the anxiolytic effects of estrogen through using animal models. High levels of endogenous estrogen can reduce anxiety and depression-related behavior [24]. Evidence suggests that estrogen regulates CRH in the hypothalamus. In fact, estrogen minimizes the synthesis of CRH by affecting the neurons located in the hypothalamus [25]. It is noteworthy that trans-anethole has steroidogenic properties. Estrogen also plays a role in reducing CRH. Therefore, it is assumed that trans-anethole like estrogen has a protective effect on CRH neurons and exerts its anti-stress effects by inhibiting the synthesis of this neuropeptide.

The hypothalamic-pituitary-adrenal (HPA) axis is a neuroendocrine system that controls the stress response. [26]. The HPA axis as biological system is preactivated under stressful conditions. Hyperactivation of the HPA axis due to the release of cortisol or corticosterone (rat) into the circulation in response to stress can affect health [27]. There is a close relationship between cortisol and noradrenaline. Increase in cortisol levels can lead to an increase in noradrenaline activity [28]. Also, the results showed that cortisol increases the expression of CRH [29]. Acute stress immediately leads to the activation of the CRH signal. Then, a cascade of hormones is produced to respond to stress [30]. The present research indicated that trans-anethole reduces CRH gene expression. Probably, trans-anethole, due to its antioxidant properties, inhibits the activity of the HPA axis and reduces the release of cortisol, leading to a decrease in noradrenaline levels. Therefore, one of the possible mechanisms of stress reduction by trans-anethole is the inhibition of noradrenaline release, which in turn reduces CRH gene expression.

Calcitonin gene-related peptide (CGRP) is a neuropeptide of 37 amino acids that is distributed in the different areas of the brain, including the hypothalamus, amygdala, and hippocampus. CGRP plays a role in vasodilation, pain, energy, regulation of anxiety and depression-related behavior [31]. *Pimpinella anisum* (anise) has various compounds including trans-anethole. Trans-anethole is the main compound of anise. Studies have shown that anise oil stimulates the GABArgic system [32]. Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter that plays an important role in controlling synaptic stimulation /inhibition [33]. GABA's inhibitory effect on GCRP level has been reported [34]. Therefore, it is possible that trans-anethole, by increasing the activity of the GABArgic pathway, leads to inhibiting the level of GCRP expression and ultimately reducing stress.

Several studies have shown that acute stress increases significantly the inflammatory factors and oxidative stress. Stress increases the levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) [35]. inflammatory factors stimulate the release of CGRP [36, 37]. Trans-anethole is the main combination of anise and fennel. Studies have demonstrated that trans-anethole has various medicinal activities, including neuroprotective, antioxidant, anti-inflammatory, estrogenic, and anticancer effects [37, 38]. In the present study, it is suggested that trans-anethole can exert anti-stress effects by reducing the expression of CGRP through inhibiting the production of inflammatory factors.

5. Conclusion

In summary, the present results showed that the administration of trans-anethole at doses of 150 or 250 mg/kg improved stress-induced behaviors in rats. Trans-anethole downregulated the hypothalamic *CGRP* and *CRH* gene expression. The decrease of hypothalamic *CGRP* and *CRH* gene expression may be the central mechanisms by which trans-anethole exerts its anxiolytic effect in stress model rats. The present results may suggest the trans-anethole be the potential anxiolytic target for stress management.

Ethical Considerations

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Author's contributions

Authors' contributions All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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