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The Protective Effect of Hydroalcoholic Extract of Green Tea (*Camellia sinensis* L.) on Anxiety Behaviors Induced with Chronic Noise Stress in Adult Male Wistar Rats

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Abstract

Background: The recruitment of medicinal herbs for the treatment of psychological disorders such as anxiety and stress has a long history. The purpose of the present study was to investigate the compensatory effects of hydroalcoholic extract of green tea on anxiety behaviors that are induced by noise stress in male Wistar rats.

Methods: In this study, male Wistar rats weighing 200±20 g were randomly divided into six groups (n = 10): 1) control group, 2) sham- noise stress (two times per day and each time for 15 minutes, for 21 days, without noise stress), 3) chronic noise stress (two times per day and each time for 15 minutes, for 21 days, at 87.3 dB(A) and the frequency of 80 kHz), 4) Green tea (50, 100, 150 mg/kg i.p. for three weeks), 5) sham- green tea with saline injection for 21 days, 6) noise stress + green tea extract (50, 100, 150 mg/kg i.p. for three weeks). Their serum cortisol levels was measured by ELISA test before and after the tests. Behavioral tests were performed including elevated plus-maze, territory discrimination, and passive avoidance test. All statistical analyses were performed using SPSS software.

Results: Serum corticosterone levels significantly decreased in noise stress groups treated with green tea compared to the control group ($P<0.05$). Based on the passive avoidance test, the time spent in the dark box showed a significant decrease in the noise stress group treated with green tea (150 mg/kg) compared to the noise stress group. Based on the territory discrimination test, the delay for entering the foreign segment in the green tea treated-groups showed a significant decrease ($P<0.05$) as compared to the noise stress group. The results of the elevated plus-maze test showed a significant increase in the time spent on the open arms in green tea extract-treated groups in comparison with noise stress group.

Conclusion: Findings show the unique properties of green tea extracts in reducing serum corticosterone levels and improvement of anxiety behaviors caused by noise stress in animals. In addition, the useful effects of herbal medicine on anxiety disorders were confirmed in our study.

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Introduction

In today's modern world, the various types of stress and pollution are inevitable phenomena that affect the body systems. Majority of the people are exposed to potentially dangerous noise levels such as noise from the workplace, urban traffic, aircraft and home appliances (1,2). The impacts of noise on individuals vary according to age, gender and physical health. The brain recognizes the sound levels and distinguishes between different voice tensions. In addition to auditory damage, chronic exposure to noise pollution causes behavioral and psychophysiological disorders including anxiety (3). Anxiety is one of the most common psychiatric disorders and ameliorates with the improvement of the socioeconomic situation. This disorder can be primary or secondary, which is caused by other physical or mental illnesses, especially depression (4). There are many psychiatric anxiety disorders, including: phobia, general anxiety, social anxiety, obsessive-compulsive disorder, post-traumatic stress, and specific anxiety (2). Noise pollution is associated with adverse effects on the central nervous system (CNS). With regard to the mechanisms involved in anxiety, researchers in biological sciences, neuroscientists, and psychologists have done many investigations to introduce several effective chemical and herbal drugs (5).

Green tea is made from the leaves and sprouts of the *Camellia sinensis* plant, which is considered the most consumed drink after drinking water. Green tea is a non-fermented product (6) that contains a large number of catechins. Catechins are potent antioxidants *in vitro* and *in vivo*. In addition, it contains some minerals and vitamins that increase the antioxidant potential of this tea sample (6). Green tea is also rich in anti-inflammatory and anti-cancer compounds (7). Each cup of green tea contains 50-100 mg of polyphenols and 10-50 mg of caffeine with a sedative effect. Flavonoids, theanine and aromatic compounds are other ingredients of green tea (8). Previous studies have shown that there is a relationship between tea consumption and behavioral functions in elder people, so that drinking 2 cups or more of green tea per day reduces the incidence of mental disorders. Green tea strengthens and stimulates the activity of neurons, especially in memory-related areas. In addition, drinking of green tea strengthens the nervous system and improves stress-related disorders (9). L-theanine, as an amino acid in tea, suppresses psychosocial and physiological stress in human and mice (10). Recent studies have shown that theanine can reduce the stress by inhibiting cortical neuronal stimulation in humans or by affecting the neurotransmitter secretion and function in CNS (11).

Therefore, considering the harmful effects of loud noise on the nervous system and the beneficial effects of green tea, the purpose of this study was to evaluate the effect of hydroalcoholic extract of green tea on anxiety behaviors triggered by noise stress in adult male rats.

Material and Methods

Animals and Experimental Design

In the present study, 100 male Wistar rats weighing 200 ± 20 g were used. The animals were kept under 12 hours light-dark cycle in an ambient temperature of 22±24°C and were housed in the standard cages. They had access to rat chow and water with no limitation. Then, they were randomly divided into six groups and each group contained 10 rats. In the sham-noise stress group, the animals were caged with a silent speaker

two times per day and each time for 15 minutes, for 21 days. In the second group, the animals were exposed to chronic noise stress (two times per day and it took 15 minutes in each time, for 21 days, at 87.3 dB(A) and the frequency of 80 kHz). This noise intensity was generated by computer software (NCH Tone Generator 3.26) and delivered to a loudspeaker, which was located at 30 cm from the animal's cage. The noise intensity was monitored by a sound level meter (Smart Tools co., Ltd., Japan). In the green tea groups, the animals received the extract of tea for 21 days with 50, 100 and 150 mg/kg body weight. Together with stress induction, experimental animals were treated with green tea extract for three weeks. In this regard, animals in the present study were divided into six groups (n=10) as follows:

1) Control: healthy rats without any stress and injection.

2) Sham- noise stress (two times per day and each time for 15 minutes, for 21 days, without noise stress).

3) Chronic noise stress: animals were exposed to noise stress (two times per day and each time for 15 minutes, for 21 days, at high doses of 87.3 dB(A) and the frequency of 80 kHz).

4) Sham- green tea group: the animals of this group received saline (solvent of green tea extract) intraperitoneally (i.p.) for 21 days and during this time, the animals were caged with a silent speaker.

5) Green tea (50, 100, 150 mg/kg i.p. for three weeks).

6) Noise stress + green tea extract (50, 100, 150 mg/kg i.p.): At the same time as inducing stress, the animals of this group received intraperitoneally 50, 100 and 150 mg/kg of hydroalcoholic extract of green tea for 21 days.

After inducing anxiety by chronic noise stress and receiving the extract, immediately the blood samples were taken from the hearts of the animals and samples were collected at tubes and were kept at 3500 rpm. Centrifugation was 10 minutes and the blood serum was prepared. Sampling was done at 7 am and serum levels of corticosterone were measured by ELISA kit (Abcam, USA).

Elevated plasma levels of cortisol hormone were considered as an indication of anxiety, whereas, reduction in plasma levels of these hormones was indicative of a lack of anxiety. Animal anxiety behaviors were also evaluated by three methods, including passive avoidance test, territory discrimination test and elevated plus-maze.

Green Tea Hydroalcoholic Extract Preparation

Green tea specimens were collected from the Lahijan farms of Guilan province in Iran and Scientific identification and name (*Camellia Sinensis* L.) was confirmed by a Botanist at Faculty of Agriculture, Razi University of Kermanshah.

The collected samples were dried in shadow and their leaves were separated from the stems and then were mechanically powdered. For the preparation of a hydroalcoholic extract, 200 g of green tea powder was soaked with an equal ratio of ethanol and water (1500 ml of each) for 48 hours. After passing through the filter, the resulting solution was evaporated and dried in constant air flux. After that, the dried extract was collected and used to prepare the desired dose of hydroalcoholic extract of green tea.

Behavioral Tests of Experimental Animals

Passive Avoidance Test

This test device consists of one 91×20×20 cm plexiglass box. This box is divided by a guillotine door into light (31 cm) and dark (60 cm) compartments. Stainless steel bars are located on the floor of both compartments. The room was lit by two incandescent lamps so that brightness of the light side of each box averaged 65 lx. Rodents prefer to spend most of their time in the dark. Rats with more anxiety spent more time in the dark side of the box and animals with less anxiety spent approximately similar time in both dark and light compartments. The validity of a light-dark box in rodents has been proven as a measurement method for determining the effects of anxiolytic and anxiogenic compounds in humans. To perform this test, animals were placed in the light part of the device. Then, time spent in the dark and light compartments were evaluated for 5 minutes (12).

Territory Discrimination Apparatus

This test device consists of two 40×40×40 cm wooden boxes, which are located on the sides of a starting box (20×40×40 cm) and are connected to it by corridors (15×15×15). The box in which the animal has spent its time during the 24-hour period is his personal territory and the other box is the foreign territory. The territory discrimination test involves determining the animal's ability to enter and search for a new territory, while accessing its own territory. Before starting the test, the animals were placed in the starting box for 2 minutes, and then its doors were opened and the animals could search all of the device locations within 15 minutes (13). The experiments were carried out in a calm situation and in a bright lab between 9-12 A.M. The animals' behavior was captured by a camera (Sony, Japan), which was mounted vertically at the top of the device. These videos were then blindly seen by two people. The delays in entering the personal and foreign territories and number of entries and time spent in each compartment were measured.

Elevated Plus-Maze Apparatus

This plexiglass, plus-shaped apparatus was elevated to a height of 50 cm and consisted of two 50×10 cm open arms, and two 50×10×40 cm closed arms, each with an open roof. The rats were placed in the center of the maze, and the light was supplied by a 100-watt bulb at a height of 120 cm from the center of the maze. Following their respective treatment, rats were placed individually in the center of the plus-maze, facing one of the open arms. Behavioral data were recorded using a digital camera. These parameters were measured: (a) time spent in the open arms and (b) the number of entries into the open arms during the 5-min test period. Arm entry is defined as all four paws entering an arm. The maze was cleaned with 70% ethanol after each rat was tested. The presence of rats in the center of the maze and open arms indicated that the animals had no anxiety and time spent in the closed arms were considered as an anxiety marker (14). Accordingly, the longer time spent on the open arms and a greater number of entries into the open arms were indicative of anxiolytic effects of green tea extract.

Statistical Analysis

All data are shown in mean ± SE. Kolmogorov-Smirnov test was used to assess the normal distribution of variables. Statistical analysis was performed using one-way ANOVA

followed by Tukey multiple comparison post hoc tests. Differences were considered to be statistically significant when *P* was <0.05. All statistical analyses were performed using SPSS software version 22 for windows.

Results

Findings concerning the serum levels of corticosterone in the studied groups are shown in Table 1. Chronic noise stress significantly (*P*<0.001) increased corticosterone levels compared to the control group. In this study, no significant differences were observed between the results of the control group and the sham-noise group. Therefore, we did not include the results of the sham-noise group in our analysis.

The injection of green tea extract (50, 100 mg/kg) after receiving noise stress reduced the level of this hormone compared to the chronic noise stress group and this decrease was statistically significant (P<0.01), but it was at a higher level compared to the control group. The group treated with hydroalcoholic extract of green tea (150 mg/kg) showed a significant decrease (P<0.001) compared to the noise stress group. In this regard, the hormone level was very close to the normal range. To demonstrate the effect of green tea on the level of cortisol in plasma, it was also shown that green tea extract did not indicate any significant difference compared to the control group.

Table 1. The effect of intraperitoneal injection of green tea hydroalcoholic extract and 21 days of exposure to the noise stress on serum corticosterone concentration. Each column represents mean ± SE for 10 rats.

Groups	control	Noise stress	GT 50mg/kg	GT 100mg/kg	GT 150mg/kg	Noise stress +50mg/kg GT	Noise stress +100mg/kg GT	Noise stress +150mg/kg GT
Serum Corticosterone levels (µg/dL)	3.75±0.17	***8.825±0.27	4.1±0.07	4.25±0.18	3.24±0.5	***,##7.44±0.46	***,##5.87±0.33	###4.69±0.21

***P< 0.001 represent significant difference with the control group. ##P<0.01 and ###P<0.001 represent significant difference between the green tea-treated groups with noise stress group. GT = green tea extract.

Passive Avoidance Test

The time spent in the dark and light chamber in the studied groups is shown in Figure 1. Animals of the noise stress group spent more time in the dark chamber than the control animals. The time spent in the dark chamber in the groups treated with green tea extract was reduced compared to noise stress, and this decrease was statistically significant for the 150 mg/kg dose of green tea extract.

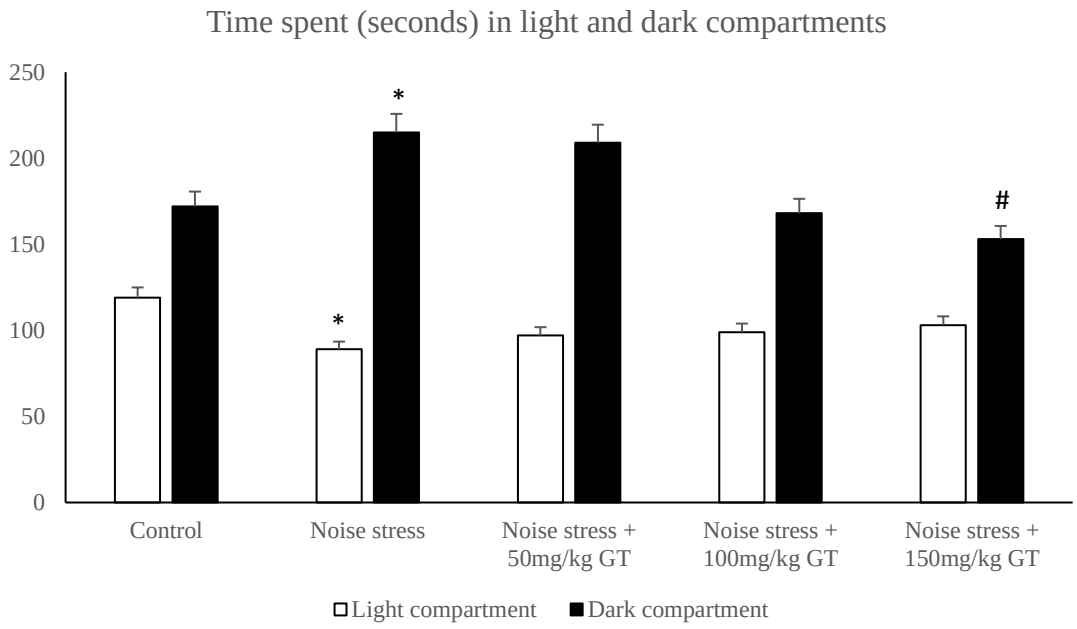

Figure 1. The effect of intraperitoneal injection of green tea hydroalcoholic extract and 21 days of exposure to the noise stress on time spent (seconds) in light and dark compartments in passive avoidance test.

*P< 0.05 represents a significant difference with the control group. #P< 0.05 represents a significant difference with the noise stress group. Each column represents mean ± SE for 10 rats.

Territory Discrimination Test

The results of territory discrimination test showed that the delay in entering the foreign part was more than the delay in entering the personal part in the noise stress compared to the control group. The delay in entering the foreign territory in the group treated with green tea extract (100 mg/kg) was significantly lower than that in the noise stress group. In the group treated with 150 mg/kg of green tea extract, delay in entering the foreign territory showed a significant decrease compared to the noise stress and this delay was very close to the control levels (Figure 2A). The number of entry into the personal and foreign territory was compared in all groups (Figure 2B). In the treated groups, the number of entry into the foreign territory was lower than that in the control group, but it was not significant. Time elapsed in the foreign territory in the treated groups increased significantly ($P<0.05$) as the amount of extract was added (100,150 mg/kg) compared with the control group (Figure 2C). In the noise stress group, the time spent in both personal and foreign territory, in comparison with the control group, was significant ($P<0.01$), but it was lower in both territories than in the treatment groups. Data of green tea group were eliminated from the chart because they did not have a significant difference with the control group.

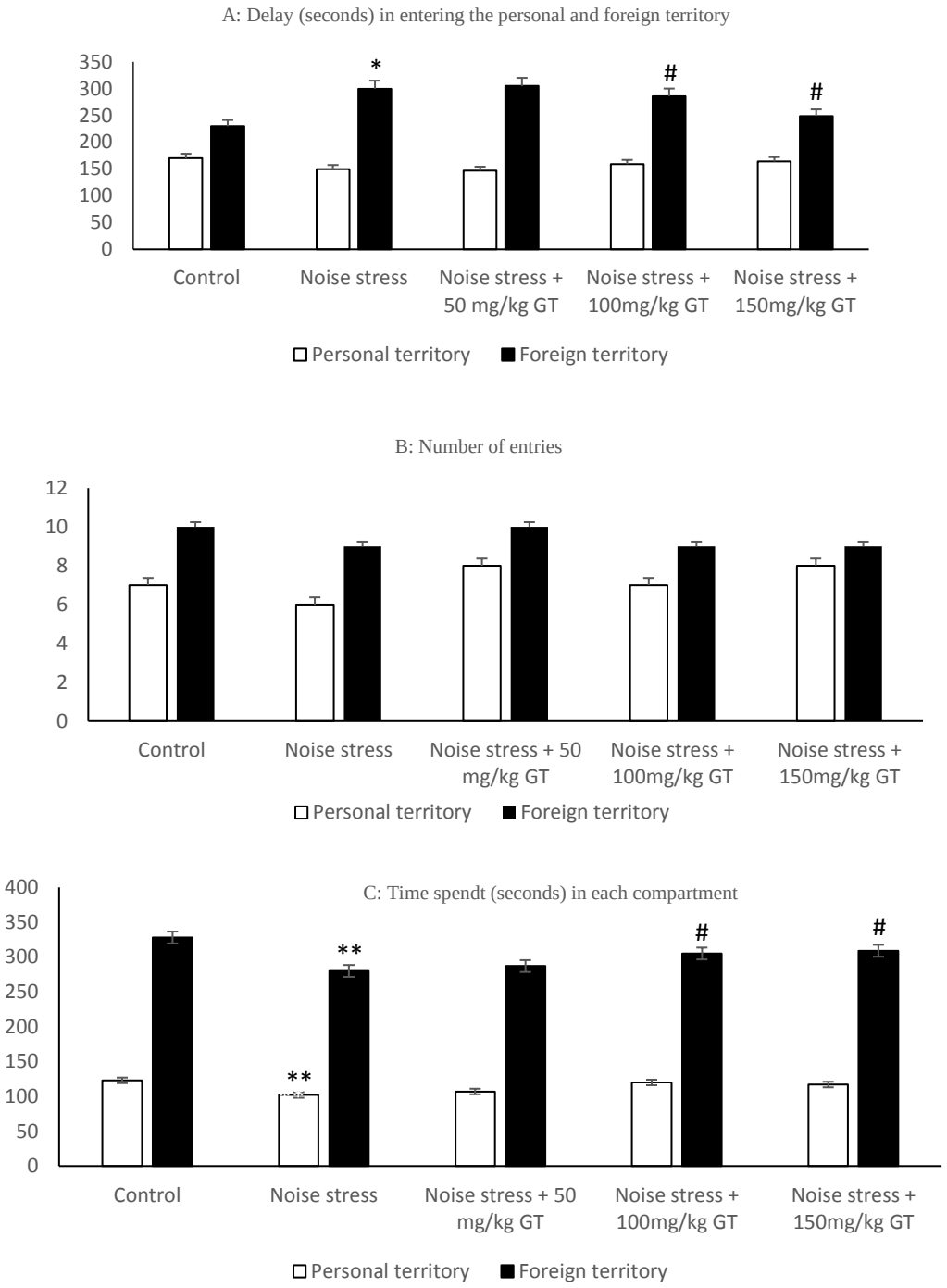

Figure 2. A: The effect of intraperitoneal injection of green tea hydroalcoholic extract and 21 days of exposure to the noise stress on delay in entering the personal and foreign part in territory discrimination test, B: The mean number of entries in the personal and foreign territory, and C: The mean time spent in the personal and foreign territory.

*P< 0.05 and **P< 0.05 represent significant differences with the control group. #P< 0.05 represents a significant difference with the noise stress group. Each column represents mean ± SE for 10 rats.

Elevated Plus-Maze Test

The level of anxiety in five studied groups is shown in Table 2. Exposure to chronic noise stress for 21 consecutive days, caused a significant difference (P <0.05) in the level of anxiety (the mean of the number of entries into open arms) in the noise stress group compared to controls. In addition, the number of entries into open arms in the groups treated with green tea extract (50,100 & 150 mg/kg) was significantly (P <0.05) higher than the noise stress. About other parameters of anxiety (the mean time spent in open arms), significant differences (P <0.001, P<0.01) were also observed between noise stress with groups treated with green tea extract (100 & 150 mg/kg). A significant difference was also observed between the noise stress group compared to the control group. Since the groups receiving the green tea did not have a significant difference compared with the control group regarding the number of entries into open arms and the time spent in each arm, the data of green tea extract were removed from the table.

Table 2. The anxiety parameters in elevated plus-maze test [time spent (seconds) in open arms and the number of entries into open arms].

Groups (n = 12)	Time spent (seconds) in open arms	Number of entries into open arms
Control	71.78 ± 1.2	3.1 ± 0.1
Noise Stress	25.78 ± 1.3***	1.7 ± 0.15*
Noise Stress + Green Tea extract (50 mg/kg)	27.53 ± 1.1	1.9 ± 0.1#
Noise Stress + Green Tea extract (100 mg/kg)	50.46 ± 1.8##	2.1 ± 0.15#
Noise Stress + Green Tea extract (150 mg/kg)	75.23 ± 2.0###	2.8 ± 0.2#

Data are presented as mean ± SE. ***P< 0.001, *P<0.05 represent significant differences with the control group. #P< 0.05, ##P< 0.01 & ###P< 0.001 represent significant differences with the noise stress group (n=10).

Discussion

The findings of the present study showed that prolonged exposure to noise stress could lead to anxiety behaviors and increased corticosterone secretion in rats. The hydroalcoholic extract of green tea reduced the level of noise stress-induced anxiety by decreasing corticosterone levels and improving the animal behaviors. At a higher dose, it could greatly restore the animal behavior to the normal state.

Sound is a type of mechanical wave and is considered as an invaluable tool for communication. Conversely, undesirable and harmful levels of sound are known as noise. With fast industrialization and modernization, noise pollution in the workplace and industry has become an unavoidable public health problem (15). Noise pollution leads to many detrimental auditory and non-auditory effects (16). The chronic exposure to noise stress results in fatigue, annoyance, arousal response followed by sleep disturbance and reduction in social communications (17). To date, many experimental studies demonstrated the detrimental effects of noise stress on brain function such as memory and learning impairments (18). Release of stress hormones and mood disturbance as well as anxiety responses have been reported following exposure to the

noise stress (19). Behavioral evidence from animal studies has also revealed the harmful effects of noise stress on psychological indications (20). The results of our study showed that exposure to noise stress leads to fewer numbers of entries and less time spent in the open arms of the elevated plus maze. This indicates that animals under noise stress have more anxiety than controls. Previous studies have shown that noise stress changes the brain neurotransmitter levels, produces atrophy of dendrites, and elevates plasma corticosterone levels (21). In fact, the auditory system is permanently active even during sleep, and audiogenic stimulation is subcortically connected via the amygdala to the hypothalamic-pituitary-adrenal-axis (HPA-axis) (22). Thus, noise causes the release of different stress hormones such as corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) and activates autonomic systems of norepinephrine/locus coeruleus and its effectors (23). In the current study, serum corticosterone levels increased significantly because of the chronic noise stress compared with controls. This data confirmed the anxiety behaviors in animals.

Today, medicinal plants form an important part of traditional medicine in many countries, and they have a special place in new therapeutic approaches. Increasing evidence has expanded the role of green tea from a traditional beverage to a source of pharmacologically active molecules with diverse health benefits. Green tea leaves contain three main components which act upon human health: xanthic bases (caffeine and theophylline), essential oils and especially polyphenolic compounds (6). The balance between theanine, caffeine, and catechin determines the quality of green tea. Theanine exhibits an excellent stress-reducing effect on mice and human (24). With respect to the presence of flavonoids in the green tea extract, it is likely that this plant will produce sedative and anxiolytic effects via impact on benzodiazepine receptors bound to GABA-A receptors (8). The results of the current research indicated that green tea extract treatment diminished the noise stress-induced anxiety behaviors. Groups treated with green tea extract had more entries and they spent more time in the open arms of the elevated plus maze device. This caused a significant reduction in anxiety compared to the noise stress group. Generally, rodents have an instinctive sensation and a tendency to enter all of the arms, and on the other hand, they have fears and anxieties in open and light environments and spend more time in the closed arms. Therefore, less entry and less time spent in open arms indicate increased anxiety in the animals (25). Reduced anxiety behaviors in the groups treated with green tea extract may also be due to the decreased levels of corticosterone and have an effect on neuroendocrine pathways. Uygur and Arslan reported that noise stress on one-month-old Sprague Dawley rats (45 minutes daily, 100 dB intensity for 3 weeks) did not affect cognitive functions and anxiety-related behaviors (26). The results of this study are not consistent with our results. This discrepancy can be attributed to the age and race of the studied rats, the intensity and duration of noise stress, and the tests used to assess anxiety. In another study, it was reported that noise stress (4 hours per day, the intensity of 100 dB for 15 days) increased anxiety behaviors of male Wistar rats in the elevated plus maze test (20), which was similar to our findings.

In addition, the results of territory discrimination test showed that rats of the noise stress group had a delay in entering the foreign area than the control group. This indicates a weaker social reaction and more anxiety in these animals. Treatment with green tea extract

especially a higher dose (150 mg/kg) decreased the delay time in entering the foreign territory.

In general, the mechanism by which exposure to noise stress affects psychological status is very complicated and requires more detailed studies. Furthermore, confirmation of the potential effects of green tea will also require the separation and identification of the chemical structure of plant constituents. More specific research on each of these constituents needs the use of different animal models to determine the mechanism of its sedative and anxiolytic effects.

Conclusion

Noise is a displeasing and inescapable stressor, which affects neurotransmitters and hormone systems and induces changes of various neurophysiological responses. Considering the results of the current study, it can be concluded that observed behavioral deficits following chronic noise stress might be associated with an imbalance of stress-related systems such as HPA-axis, which potentially results in anxiety. Green tea extract with its useful substances that affects the nervous system can greatly relieve the levels of anxiety caused by noise stress and resists the animals against anxiety.

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Conflict of interest

The authors declare that they have no conflict of interest.

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