Effects of melatonin and theanine administration on pentylenetetrazole-induced seizures and brain tissue oxidative damage in ovariectomized rats

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Background/aim: The effects of coadministration of melatonin and theanine (Mel/Thea) on pentylenetetrazole (PTZ)-induced seizures and brain tissue oxidative damage were investigated in ovariectomized (OVX) and sham-operated rats.

Materials and methods: The rats were divided into the following groups: 1) sham, 2) ovariectomized (OVX), 3) sham-PTZ, 4) OVX-PTZ, 5) sham-Mel/Thea-PTZ, and 6) OVX-Mel/Thea-PTZ. Groups 1–4 received saline, while groups 5 and 6 received a combination of Mel/Thea for 6 weeks. All animals except for those in groups 1 and 2 received a single injection of PTZ.

Results: The OVX-PTZ group had higher generalized tonic-clonic seizure (GTCS) latency compared to the sham-PTZ group. Administration of Mel/Thea increased minimal clonic seizure and GTCS latencies in both the sham-Mel/Thea-PTZ and OVX-Mel/Thea-PTZ groups compared to the controls. Additionally, PTZ exposure increased malondialdehyde levels and reduced thiol concentrations in brain tissues of both the sham-PTZ and OVX-PTZ groups. Mel/Thea pretreatment resulted in MDA reduction and thiol increase in brain tissues.

Conclusion: The results of this study demonstrated the antioxidant and anticonvulsant activities of Mel/Thea despite the presence or absence of ovarian hormones.

Key words: Melatonin, theanine, pentylenetetrazole, seizures, oxidative stress, brain, rat

1. Introduction
Epilepsy is a common neurological disease that affects approximately 1% of the population (1). It is characterized by abnormal episodic bursts of electrical activity in neurons, which significantly impact the cognitive processes and behavior of the affected patients (2). The central nervous system (CNS) is very susceptible to oxidative injury due to high levels of membrane lipid constituents (3,4). Accumulating evidence has revealed an important role for oxidative stress, both as a consequence and as a cause of epileptic seizures (5). It has been found that prolonged seizures increase the production of free radicals (6) and result in oxidative damage to lipids, DNA, and susceptible proteins (7). Moreover, it has been shown that reactive oxygen species (ROS) may underlie the neurotoxic effects of some convulsant agents, such as pentylenetetrazole (PTZ) (8). Furthermore, it has been demonstrated that the antioxidative properties of melatonin, vineatrol, trans-resveratrol, and alpha-lipoic acid are associated with their anticonvulsant effects (6,9). Collectively, it appears that using antioxidants is a promising approach in the development of new therapeutic agents with neuroprotective effects against epileptic seizures (5,7).

Additionally, it has been well documented that female sex hormones play a role in epilepsy (10,11). There are also close communications and connections between the hypothalamus and pituitary gland, which regulate sex hormone secretion, and the temporal lobes, which
are important areas in seizures (12). Female epileptics frequently show exacerbated symptoms at specific points during their menstrual cycle, for example during periods of low progesterone levels (10). It has also been found that progesterone reduces neuronal activity, while estrogen increases neuronal excitability (11). In fact, previous studies demonstrated that low progesterone levels are associated with increased seizure frequency in women (13). Elevated estrogen levels during perimenopause also appear to exacerbate epilepsy (13). It has also been observed that testosterone and its metabolites have antiseizure effects in men (14). These findings collectively suggest the potential influence of sexual hormones on seizure susceptibility.

On the other hand, some dietary components with antioxidative properties have shown neuroprotective effects. For instance, L-theanine (gamma-glutamyl-L-ethylamide), a unique amino acid present almost exclusively in tea, has demonstrated antioxidative and neuroprotective effects (15,16). Previous studies have shown that L-theanine increases brain serotonin, dopamine, and gamma amino butyric acid (GABA) levels; improves memory function; and has a neuroprotective effect in several animal models of neurological disorders (17,18).

Additionally, some neuroagents with antioxidative properties, such as melatonin, were demonstrated to have neuroprotective effects in previous studies (19–23). Melatonin, an indoleamine derivative of serotonin produced by the pineal gland, has shown antioxidative, sedative, anticonvulsive, and hypnotic effects in the CNS of mammals (19,21–23). In rats, melatonin has anticonvulsant and inhibitory effects on the glutamate-mediated response of the striatum to motor cortex stimulation (20). Melatonin also depresses brain excitability and prevents seizures (6). It has been well documented that melatonin influences reproductive hormones both in males and females (24,25). It has been shown that melatonin decreases estrogen levels while increasing the progesterone concentrations in rats (26).

The objective of the present study was to evaluate the effects of the administration of a combination of melatonin and theanine on seizures and oxidative brain tissue damage induced by PTZ in ovariectomized (OVX) rats.

2. Materials and methods
2.1. Drugs and chemicals
PTZ, melatonin, and theanine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemical compounds such as thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB), ethylenediaminetetraacetic acid (EDTA), and hydrochloric acid (HCl) were bought from Merck (Kenilworth, NJ, USA).

2.2. Animal groups
Forty-eight virgin female Wistar rats (230 ± 20 g in weight) were maintained in an animal house under controlled conditions, including 12-h light/dark cycle, 22–24 °C temperature, and appropriate humidity, with laboratory chow and water provided ad libitum. The study protocol using the laboratory rats complied with the general guidelines of animal care of Mashhad University of Medical Sciences, Iran.

The animals were aclimatized for 15 days and then were ovariectomized under ketamine anesthesia (150 mg/kg, i.p.) (27–30). Anesthesia was confirmed by a reduced respiratory rate and a lack of response to the gentle pinching of the foot pad. Abdominal incisions were made through the skin of the flank of the rats, and the ovaries and ovarian fats were removed. Ovaries were isolated by ligation of the most proximal portion of the oviduct before removal. The same procedure was performed on the rats in the sham group, except that the wound was closed without removing the ovaries (31–33).

Animals were divided into the following 6 groups (n = 8): 1) sham, 2) OVX, 3) sham-PTZ, 4) OVX-PTZ, 5) sham-Mel/Thea-PTZ, and 6) OVX-Mel/Thea-PTZ. The animals in groups 1–4 were treated with saline daily (2 mL/kg) for 6 weeks, while groups 5 and 6 received a combination of melatonin and theanine (Mel/Thea; 3 mg and 25 mg/kg) intraperitoneally daily for 6 weeks. After 6 weeks of saline or Mel/Thea injections, animals in groups 3–6 received a single injection of PTZ (90 mg/kg, i.p.). Minimal clonic seizure (MCS) and generalized tonic-clonic seizure (GTCS) latencies, as ictal behaviors, were then measured (34–37). Finally, the animals were sacrificed, and the cortical and hippocampal tissues were removed for biochemical analyses.

2.3. PTZ-induced seizures
In order to assess the ictal behavior, animals were placed in a Plexiglas arena (30 cm × 30 cm × 30 cm) after PTZ injection and were observed for 60 min after PTZ administration. Behavioral responses of the animals to PTZ were evaluated using the following criteria: latency to the first MCS, incidence of MCS, latency to the first GTCS, incidence of GTCS, and mortality percentage (34–36).

2.4. Biochemical assessment
After behavioral assessments, the animals were sacrificed and the brains were removed. The cortical and hippocampal regions were dissected on an ice-cold surface and homogenized in ice-cold phosphate-buffered saline to give 10% homogeny. Total SH groups were measured using DTNB as the reagent. This reagent reacts with the thiol groups to produce a yellow complex that has peak absorbance at 412 nm. Briefly, 1 mL of Tris-EDTA buffer (pH 8.6) was added to 50 µL of brain homogenate in 1-mL cuvettes, and sample absorbance was read at 412
nm against Tris-EDTA buffer alone (A<sub>1</sub>). Then 20 µL of DTNB reagent (10 mM in methanol) was added to the mixture. The mixture was incubated at room temperature for 15 min, and then the sample absorbance was read again (A<sub>2</sub>). The absorbance of DTNB reagent was also read as the blank (B). Total thiol concentration (mM) was calculated using the following equation (37–40):

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\text{total thiol concentration (mM)} = \frac{(A_2 - A_1 - B) \times 1.07}{(0.05 \times 13.6)}.
\]

Malondialdehyde (MDA) levels, as an index of lipid peroxidation, were measured in cortical and hippocampal regions. MDA reacts with TBA as a thiobarbituric acid reactive substance to produce a red-colored complex that has peak absorbance at 535 nm. The TBA/TCA/HCL reagent was added for homogenization, and the solution was incubated in a boiling water bath for 40 min. After cooling, the whole solution was centrifuged at 1000 × g for 10 min. The absorbance was measured at 535 nm (37–40). The MDA concentration was calculated as follows: C (M) = absorbance/(1.56 × 10<sup>5</sup>.

2.5. Statistical analysis
All data were expressed as mean ± standard error of the mean (SEM) and were analyzed using one-way ANOVA followed by Tukey’s post hoc comparison test. Comparisons between two groups were done using an unpaired t-test. P < 0.05 was considered to be statistically significant.

3. Results

3.1. Behavioral results
PTZ exposure was associated with seizure incidence (MCS and GTCS) in all PTZ-exposed groups. Ovariectomy showed a protective effect in PTZ-induced seizures by increasing the GTCS latency in the OVX-PTZ group compared to sham-PTZ group (P < 0.01, Table 1). However, it did not affect the MCS latency (P > 0.05, Table 1). Additionally, Mel/Thea administration significantly increased both MCS and GTCS latencies in the sham-Mel/Thea-PTZ group compared to the sham-PTZ group (P < 0.05, Table 1). Mel/Thea treatment had a similar effect on OVX rats, as it increased the MCS and GTCS latencies in the OVX-Mel/Thea-PTZ group compared to the OVX-PTZ group (P < 0.01, Table 1).

3.2. Biochemical results
Ovariectomy was associated with biochemical changes in brain tissue, which was seen in the reduced thiol contents and increased MDA levels in the hippocampal regions of the OVX group compared to the sham group (P < 0.001, Table 2). PTZ exposure also resulted in a significant reduction in thiol levels and a significant increase in MDA concentrations in the hippocampal tissues of the sham-PTZ group compared to the sham group (P < 0.01 and P < 0.001, respectively, Table 2). Additionally, PTZ exposure aggravated the adverse effects of ovariectomy on hippocampal thiol concentrations by reducing the hippocampal thiol levels of the OVX-PTZ group compared to the OVX group (P < 0.01); however, it did not cause a significant difference in MDA concentrations (P > 0.05, Table 2).

Administration of Mel/Thea improved the hippocampal thiol levels in the sham-Mel/Thea-PTZ group (P < 0.05), but it did not make a significant difference in hippocampal MDA concentrations between the sham-Mel/Thea-PTZ and sham-PTZ groups (P > 0.05, Table 2). Furthermore, Mel/Thea administration was associated with the alteration of both MDA and thiol levels in the hippocampal area of the OVX-Mel/Thea-PTZ group by reducing MDA levels (P < 0.05, Table 2) and increasing thiol concentrations compared to the OVX-PTZ group (P < 0.001, Table 2).

In addition to biochemical alterations in the hippocampal area, ovariectomy also caused significant changes in MDA and thiol levels in cortical regions. OVX rats showed reduced thiol and increased MDA levels in cortical areas compared to the sham group (P < 0.05 and P < 0.001, respectively, Table 3). Similarly, PTZ exposure was associated with an increased level of cortical MDA and a reduced level of cortical thiol in both the sham-PTZ and OVX-PTZ groups compared to their control groups (sham and OVX groups, respectively) (P < 0.05, Table 3).

<table>
<thead>
<tr>
<th>Groups</th>
<th>MCS latency (s)</th>
<th>GTCS latency (s)</th>
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<tbody>
<tr>
<td>Sham-PTZ</td>
<td>59.4 ± 4.05</td>
<td>67.6 ± 5.48</td>
</tr>
<tr>
<td>Sham-Mel/Thea-PTZ</td>
<td>135 ± 24.09*</td>
<td>227.66 ± 77.73*</td>
</tr>
<tr>
<td>OVX-PTZ</td>
<td>105.88 ± 24.8</td>
<td>144.33 ± 32.75**</td>
</tr>
<tr>
<td>OVX-Mel/Thea-PTZ</td>
<td>262.2 ± 67.109**</td>
<td>416.2 ± 120.41**</td>
</tr>
</tbody>
</table>

All data were expressed as mean ± SEM (n= 8 in each group).

*P < 0.05, **P < 0.01 compared to sham-PTZ.
**P < 0.01 compared to OVX-PTZ.
Mel/Thea administration improved both MDA and thiol levels in the cortical regions of the sham-Mel/Thea-PTZ group compared to the sham-PTZ group (P < 0.001 and P < 0.05, respectively, Table 3). Similarly, the Mel/Thea combination reduced the cortical MDA concentrations while increasing thiol levels in the OVX-Mel/Thea-PTZ group compared to the OVX-PTZ group (P < 0.05 and P < 0.001, Table 3).

4. Discussion
Oxidative stress is involved in many neurological and neurodegenerative disorders. Previous studies have demonstrated an increase in free radical levels during seizures (6), suggesting the important role of oxidative stress in the pathogenesis of epileptic seizures (5). Similarly, in the present study, we observed an increase in MDA levels and a reduction in total thiol groups in the brain.
tissues of animals that had PTZ-induced seizures. It has been well demonstrated that production of ROS, including superoxide anions, hydroxyl radicals, and hydrogen peroxide, increases in the brains of animals subjected to seizures (41,42). Oxidative damage of brain tissue by free radicals may lead to psychiatric or cognitive problems such as depression, anxiety, and memory loss (43,44). The reduction of the life span observed in epileptic patients may also be partly related to neuronal oxidative damages (45). Furthermore, oxidative stress has been suggested as a link between aging and seizures (46). The results of the present study also demonstrate a link between seizures and sex hormones. For instance, it was hypothesized that sex hormones exert their regulatory effects on neuronal excitability by modulating neurotransmitter receptors including GABA, N-methyl-D-aspartic acid (NMDA), and opioid receptors, as well as by direct and/or indirect regulation of adenosine receptors (64–67). These mechanisms may be involved in the lower susceptibility of OVX rats to PTZ-induced seizures in the present study.

Furthermore, several studies have demonstrated the anticonvulsant effect of melatonin on the sex hormones of female rats (25,68,69). We hypothesized that the effects of Mel/Thea on seizures may be different between OVX and treatment-naive female rats. For this reason, the effects of a combination of Mel/Thea on PTZ-induced seizures in OVX and naïve female rats were also investigated. The administration of Mel/Thea combination delayed latency of PTZ-induced seizures and decreased oxidative damage in both naïve female and OVX rats, without a significant difference between the two groups. It has been suggested that the anticonvulsant effects of melatonin may be due to its antioxidative function, neuroprotective effects, central GABAergic transmission, and regulation of the nitric oxide (NO) pathway (70). Mohanan and Yamamoto showed that the scavenging of hydroxyl radicals may contribute to the anticonvulsant effects of melatonin (53). In another study, Acula-Castroviejo et al. demonstrated that melatonin increased GABA concentration in epilepsy (71). Yahyavi-Firouz-Abadi et al. also reported that the NO pathway is involved in the melatonin-induced modulation of seizure susceptibility in mice (72). These mechanisms may be involved in the anticonvulsant effects of Mel/Thea in the current study.

In conclusion, the results of this study demonstrated that...
increases anticonvulsant activity. These beneficial effects are accompanied by an antioxidant effect in brain tissues. We did not find any difference in the efficiency of Mel/Tea between treatment-naive and OVX rats, but further investigations are needed.

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References


