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Original article

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Effect of Treatment with Thyme Extract on Urinary Levels of Melatonin in an Experimental Autoimmune Encephalomyelitis Mouse Model

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Abstract

Background: *Thymus vulgaris*, or thyme belongs to the *Lamiaceae* family of aromatic plant species and has established antioxidant and anti-inflammatory properties. We examined the association between thyme extract treatment to recovered urinary levels of melatonin, a hormone with neuroprotective effects, in mice induced with EAE.

Methods: Eight B6 mice induced with EAE were randomized into two groups and exposed to either 50 mg/kg of thyme extract or PBS. After EAE induction, mice were injected i.p every other day from day 0 to 21. Four B6 mice without EAE were considered the healthy control group. Urine samples were collected consecutively for two 24 h periods on day 19 and 20. We examined whether thyme extract treatment modified urinary melatonin sulfate concentration (ng/mL) in EAE-induced mice using an ELISA.

Results: The clinical score and body weight in thyme-treated EAE group were significantly lower in comparison to the EAE control group at indicated time points. The urinary melatonin concentration was significantly lower in the EAE control group compared to the healthy mice. There was no significant difference between thyme-treated and EAE groups regarding the urine melatonin concentration.

Conclusions: Our results show that exposing EAE mice to thyme extract improved their clinical symptoms, however, there was no significant effect on urinary melatonin concentration.

Keywords: Enzyme-linked immunosorbent assay (ELISA), Experimental autoimmune encephalomyelitis (EAE), Melatonin, Thymus vulgaris (Thyme), Urine.

Introduction

Multiple sclerosis (MS) is one of the most common inflammatory diseases of the central nervous system (CNS) in young adults. Previous research documents that several factors are involved in MS pathogenesis including neuronal loss due to oxidative stress, and infiltration of immune cells and neuro-immunomodulatory agents such as glucocorticoids, opioids, prolactin, and N-acetyl-5-methoxy-tryptamine (melatonin) into the CNS (1-4). The melatonin hormone is secreted from the pineal gland and regulates the body's circadian rhythm. Recent studies suggest a neuroprotective and immunomodulatory role for melatonin in inflammatory and autoimmune diseases (2, 4-6). For example, melatonin was found to modulate the immune response by decreasing T helper type 1 (Th1) responses and increasing the anti-inflammatory/Th1 ratio (7). In addition, Farez et al., found that melatonin suppressed the generation of TH-17 cells and enhanced the levels of interleukin-10 (IL-10) producing type 1 regulatory T (Tr1) cells (8). Moreover, several investigations demonstrate that melatonin levels are inversely correlated with the severity of MS symptoms, such as fatigue, insomnia and depression (1, 5, 9, 10). Interestingly, there is evidence to support that altered levels of melatonin in MS patients can be corrected after treatment with interferon beta (IFN-β) or Natalizumab (11). Common treatments for improving clinical MS include IFN-β, glatiramer

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acetate and corticosteroids. However, due to the side effects and inefficacy of these drugs, there is a growing interest in treating MS using herbal therapeutics (12). Thyme, an aromatic plant species belonging to the *Lamiaceae* family, is known to possess antioxidant and anti-inflammatory characteristics (13-17). To the authors' knowledge, there are limited publications discussing the effects of thyme extract in autoimmune diseases. Our previous research demonstrates that thyme extract has anti-inflammatory properties and can improve the clinical outcome of mice induced with experimental autoimmune encephalomyelitis (EAE), a widely used animal model of MS (18). In this study, we examined whether our previous observations correlated to urinary melatonin concentration in EAE induced mice.

Materials and methods

Mice

C57BL/6 (B6) mice (8–12 weeks old females) were purchased from Royan Institute of Isfahan, Iran. Animals were housed under controlled conditions with a 12 h day and 12 h night cycle. All experiments were approved by Animal Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran.

EAE induction

Mice were administered 200 µl of emulsion containing 400 µg myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (SBS Genetech Co. Ltd., Beijing, China) and 100 µl complete Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA) (0.4 mg of inactivated Mycobacterium tuberculosis) subcutaneously (s.c) between the shoulders and over the flank. All mice received 250 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally (i.p) on the day of EAE induction and 48 h later. Clinical signs of animals were evaluated daily and scored using a 0-8 point scale: 0=no symptoms; 1=partial loss of tail tonicity; 2=complete loss of tail tonicity; 3=flaccid tail and abnormal gait; 4=hind leg paralysis; 5=hind leg paralysis with hind body paresis; 6=hind and foreleg paralysis; and 7=death (19).

Study design

Eight B6 mice were induced with EAE and randomized into two groups, four each. EAE mice in the experimental group were exposed to thyme extract (50 mg/kg), and 2% phosphate-buffer saline (PBS) in the control group. Four mice without EAE induction was considered the healthy control group. Both EAE groups were injected i.p from day 0 to 21 every other day and sacrificed on day 22 after MOG immunization, according to the animal ethics protocol.

Extract preparation of Thyme

Thymus vulgaris (Thyme) was collected from Arac Farms (Arac, Iran) during the summer of 2016 and its identity was confirmed by the Herbarium Institute of Kerman, Iran. A hydro-alcoholic extract was prepared by drying the aerial parts of the thyme plant. Next, 100 g of thyme powder was soaked in 70% ethanol and left in the dark shaking (mildly) for 72 h. Then, the liquid phase was filtrated and concentrated in a rotary evaporator at 40°C until a semi-dried extract with 20% of the initial weight of the powder remained. Finally, the extracts were stored at -20°C and resolved in 2% PBS (18).

Urine collection

Mice were placed in cages that were equipped with a beaker under a stainless-steel grid one at a time. Urine was collected in the beaker consecutively over two 24 h periods on days 19 and 20. Samples were stored at −30°C for analysis via ELISA method. The melatonin concentration was calculated as the average of the two days.

Urinary melatonin detection

Melatonin sulfate (6-sulfatoxymelatonin) levels in urine was detected using an ELISA kit (IBL, Hamburg, Germany; cat.no. RE54031) according to the manufacturer's instructions.

Statistical analysis

Data and statistical analyses were performed using GraphPad Prism version 6.01. The data was represented as the mean ± S.E.M. We used a t-test for parametric data and a Wilcoxon signed-rank test for non-parametric data. Statistical difference is indicated as *, $P \leq 0.05$.

Results

Thyme extract treatment improved the clinical score in EAE-induced mice

After MOG immunization, the first clinical signs of EAE appeared on days 9 and 10 in the untreated and thyme-treated group, respectively. The clinical score in thyme-treated mice were significantly lower in comparison to the EAE control group at indicated time points ($P<0.05$ at day 10, and $P<0.001$ at days 13 to 21) (Fig. 1). Moreover, the body weight of mice in the thyme-treated group was significantly higher compared to untreated-EAE mice at the indicated time points ($P<0.05$ at day 13 and $P<0.001$ at days 14 to 21) (Fig. 2).

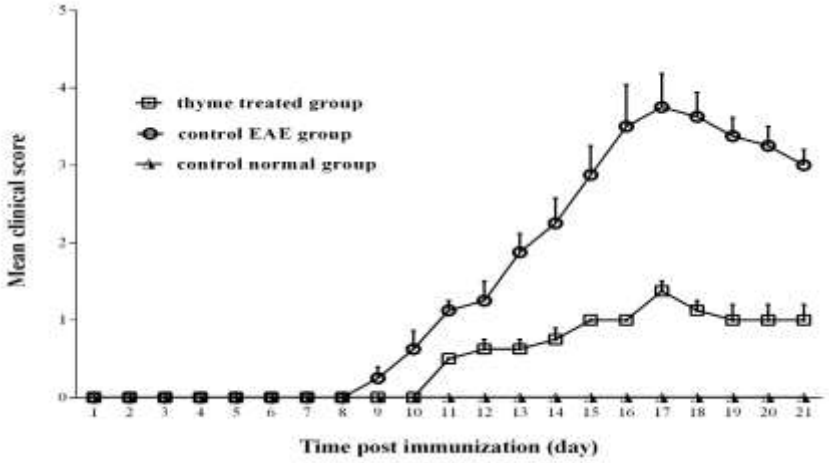

Fig. 1. The mean clinical score in EAE induced mice treated with thyme extract in comparison to the EAE control group (not treated)

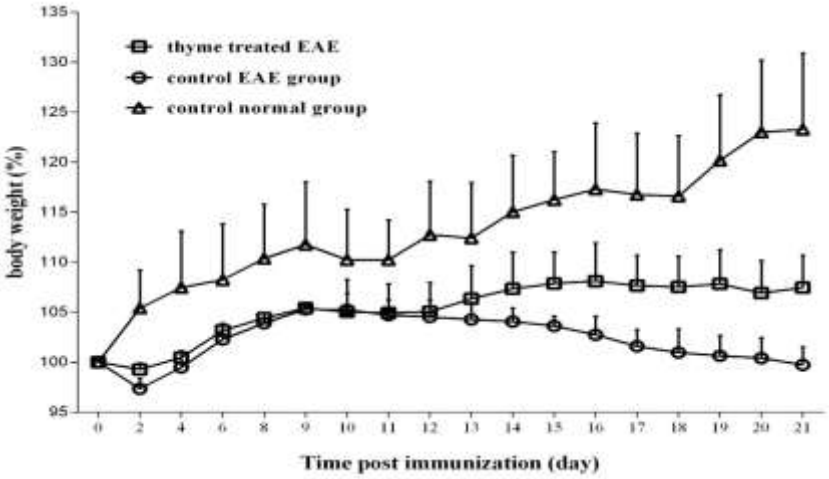

Fig. 2. The body weight of EAE induced mice treated with thyme extract in comparison to control groups (not treated)

No significant difference in urinary melatonin between thyme-treated and untreated-EAE mice

Following thyme extract treatment and clinical assessment, melatonin sulfate concentration in collected urine samples was measured in the thyme-treated EAE group, untreated-EAE control and healthy control groups. The average melatonin concentration was significantly higher in normal healthy mice compared to the untreated-EAE control group ($P=0.028$). Although melatonin levels were higher in the thyme-treated EAE group compared to the untreated-EAE group, the difference was not found to be statistically significant ($P=0.11$) (Fig. 3). Lastly, there was no significant difference between the healthy group and the thyme-treated EAE group regarding urinary melatonin concentration.

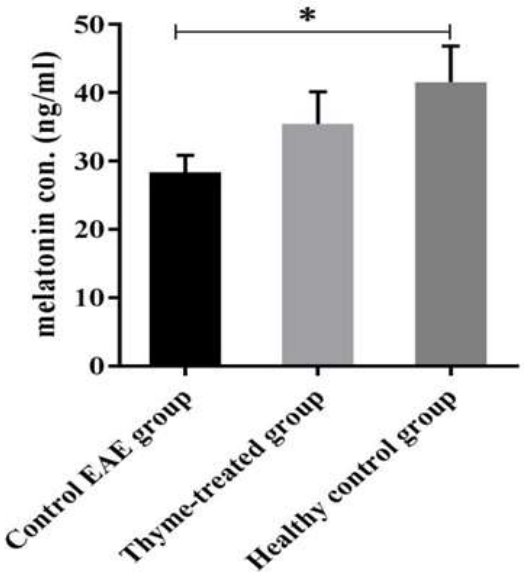

Fig. 3. The average concentration of urine melatonin sulfate levels in EAE induced mice treated with thyme extract in comparison with EAE and healthy control (not treated) groups.

Discussion

Recent studies have indicated various immunomodulatory roles of melatonin in a number of immune-related diseases including autoimmune disorders (4-6). The levels of this hormone are altered during the course of disease and is inversely related to disease severity. Therefore, finding new treatments with the least adverse effects able to modify melatonin levels could be of interest. Melatonin is present in many plants with variable levels (20,21). Therefore, treating autoimmune diseases with these plants may have promising results. This paper aimed to evaluate the effect of thyme extract treatment on melatonin sulfate levels in urine samples of EAE-induced mice. The results of this research showed that despite a decrease in melatonin in EAE-induced mice, there was no significant difference in urinary melatonin sulfate between the thyme-treated and untreated mice.

Evidence in the literature suggests an inverse correlation between the levels of melatonin and the severity of autoimmune diseases. In this study, we observed significantly lower levels of melatonin in EAE-induced control mice compared to the healthy mice. Consistent with these results, Gholipour et al. showed that lower levels of urine 6-sulphatoxymelatonin (aMT6s) was negatively associated with Functional Composite score (FCS). The FCS factor is used to assess the overall disability impact in MS patients, suggesting a novel method for assessing MS severity (4). Another study demonstrates that there is an inverse relationship between nocturnal melatonin serum levels and major depression, indicating that melatonin deficiency could lead to the development of depression in MS patients (11). In addition, some published papers have shown that there can be negative consequences from disrupting melatonin concentration and the occurrence of MS. For instance, Hedstrom et al. observed that working shift, which causes circadian disruption, could enhance the risk of MS early on (3). Melatonin depletion has also been reported in other autoimmune diseases, for example, a study by Wang et al. showed that decreased plasma melatonin levels in systemic lupus erythematosus (SLE) patients correlated with clinical manifestations (22).

Whether melatonin plays a role in improving clinical symptoms of MS is still a matter of debate. Previous investigations have reported the effect of various drugs and treatments on melatonin levels. In a study conducted on 13 MS and 12 healthy subjects, treatment with IFN-β could ameliorate decreased urinary levels of 6-sulphatoxy-melatonin (6-SMT), fatigue and sleep efficiency in MS patients (13). In addition, Natalizumab, one of the most efficient treatments for MS, increased serum melatonin levels as well as antioxidants in 18 patients with relapsing remitting multiple sclerosis (RRMS) (1). Treatment with Luzindole, an antagonist for the melatonin membrane receptor inhibited EAE development in mice following immunization with spinal cord homogenate (23). In contrast, our results found no significant difference in the levels of melatonin sulfate in the urine of thyme-treated and untreated mice. This negative result may be attributed to the selected dose of the extract. In this study, we only tested one dose of thyme extract (50 mg/kg), therefore, using a higher dose could potentially alter melatonin levels. Moreover, our findings were limited by a small sample size in each group. Finally, *Thymus vulgaris* could modulate the immune system

through other pathways not related to melatonin regulation.

In conclusion, these results demonstrated that urinary levels of melatonin were decreased in an EAE disease model. Furthermore, although treatment of EAE-induced mice with thyme extract improved disease progression of EAE, it had no significant effect on the urinary melatonin levels.

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