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Plasma Melatonin and Urinary 6-Hydroxymelatonin Levels in Patients with Pulmonary Tuberculosis

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Abstract—Tuberculosis (TB) is the second most frequent cause of death in the world, after AIDS. Delay in diagnosing TB is an important worldwide problem. It seriously threatens public health. Cell-mediated immune responses play an important role in the pathogenesis of TB infection. The course of *Mycobacterium tuberculosis* (MTb) infection is regulated by two distinct T cell cytokine patterns. Melatonin is a biomolecule (mainly secreted by the pineal gland) with free radical scavenging, antioxidant and immunoregulatory properties. Melatonin has both its direct and indirect immunomodulatory effects on the immune system. In this study, we measured plasma melatonin and urine 6-hydroxy melatonin sulphate (6-HMS) concentrations in patients with newly diagnosed TB for the purpose of investigating whether there was a relationship between their levels and MTb infection. Thirty-one newly diagnosed patients presenting with active TB and 31 healthy subjects as the control group were included in this study. Blood and 24-h urine samples were collected from all individuals. Plasma melatonin levels and urine 6-HMS were measured. Our results show that in patients with TB, mean melatonin and 6-HMS concentrations were significantly lower than in the control subjects ($p=0.037$, $p<0.001$, respectively). We believe that the treatment of TB patients with melatonin might result in a wide range of health benefits including improved quality of life and reduced severity of infection in these patients. Supplementation with melatonin may be considered as an adjunctive therapy to classic treatment of pulmonary TB, especially during the acute phase of infection.

KEY WORDS: melatonin; *Mycobacterium tuberculosis*; 6-hydroxy melatonin sulphate; pulmonary tuberculosis.

INTRODUCTION

Tuberculosis (TB) results in about 3 million deaths each year [1]. TB is the second leading cause of death worldwide, after AIDS [2]. The most common form of TB is pulmonary TB [3]. *Mycobacterium tuberculosis* (MTb) is a facultative intracellular bacterium, which can resist antimicrobial mechanisms of the cells in the immune system, including monocytes and macrophages [1]. It is well-known that bacterial, host and environmental factors influence the development of active TB [4]. In most of the cases, the host immune response controls MTb replication, and a latent infection is established. When the host immune response fails to control the MTb replication, active TB develops [5]. An effective cell-mediated immune response is crucial for controlling the infection [6]. The host response against MTb is dominated by the interaction of innate and adaptive immunity [7].

Melatonin (*N*-acetyl-5-methoxytryptamine), a highly soluble indoleamine, is derived from serotonin. Because of its unique synthesis in the pineal gland at night, it is known as the chemical expression of darkness [8]. Melatonin is

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predominantly synthesized and secreted into the blood in a circadian manner by the pineal gland during the dark phase of the light/dark cycle [9]. The peak concentrations of melatonin at night are important for establishing the difference between day and night as well as between the seasons [9, 10]. Melatonin is a potent antioxidant which may account in part for its anti-aging properties [11]. As an antioxidant, melatonin is both free radical scavenger and an indirect antioxidant due to its stimulation of antioxidative enzymes, and an immunoregulator agent [12]. Renewed attention has been given to the role of melatonin in modulating behavior, the immune system and responses to stress, cancer and aging [13, 14]. Additionally, exogenous melatonin influences circadian rhythm/sleep disorders, insomnia, cancer, neurodegenerative diseases, immune function disorders and oxidative damage [15]. Melatonin is a well-established agent which is known to exert positive effects on both the cellular and humoral immune response in mammals [16]. Melatonin exhibits both directly and indirectly its immunomodulatory effects on the immune system [17].

The major hepatic metabolite of melatonin is 6-hydroxymelatonin, of which 90 % is conjugated with sulphate and 10 % with glucuronate in the liver. These metabolites are excreted mainly in urine but also partly in bile. It has been recognized that the amount of 6-hydroxy melatonin sulphate (6-HMS) excreted in urine is a good index of melatonin synthesis and excretion [18]. Furthermore, 6-HMS also exerts antioxidant effects [19].

Although significant steps have been made in the diagnosis and treatment, the immuno-endocrine system still plays a role in controlling MTb infection, which is not fully described. To date, there are no reports on plasma melatonin or urinary 6-HMS levels in TB patients. In this study, we measured plasma melatonin and urinary 6-HMS concentrations in patients with newly diagnosed TB; the purpose was to establish a potential relationship between their levels and MTb infection.

MATERIALS AND METHODS

Study Subjects

Thirty-one newly diagnosed, randomly selected patients presenting with active TB in the Department of Pulmonary Medicine, Gulhane Military Medical Academy, Ankara, Turkey were included in this study. All patients were men and had a mean age of 22.9± 3.8 years. HIV-negative patients with active TB had an acid-fast smear or culture positive for MTb. Active TB patients were studied before or within the first 2 weeks of anti-TB treatment. In 31 patients, the diagnosis of TB was established when clinical findings were supported by histopathological evidence and after the exclusion of other known causes of granulomatosis. The control subjects consisted of 31 men healthy employees (23.6± 3.4 years) of Gulhane Military Medical Academy. By completing a questionnaire, relevant background information was provided by these volunteers and included medication, hereditary and other diseases. Verbal and written consent was obtained from all patients and control subjects, and authorization was given by the ethics committee of Gulhane Military Medical Academy, Ankara, Turkey.

Sample Preparation

Whole blood samples were collected into tubes containing ethylenediaminetetraacetate (EDTA) as anticoagulant (for preparation of plasma) and without anticoagulant (for preparation of serum) at 0800–0900 hours. Serum fraction was obtained by centrifugation (2,000×*g*, 10 min, 4 °C) after storing the whole blood at room temperature (approximately 30 min). The EDTA-containing tubes were immediately placed on ice and centrifuged (2,000×*g*, 10 min, 4 °C) within 30 min. Twenty-four-hour urine samples were collected from all individuals. No preservatives were added to the urine specimens. All plasma and urine samples were placed into tubes covered with aluminum foil and stored at −80 °C until assays.

Measurement of Melatonin

Plasma melatonin levels were measured by high performance liquid chromatography (HPLC) with fluorescence detection (Agilent Technologies 1100 Series System, Santa Clara, CA, USA), using the method defined by Ozkan *et al.* [20]. In brief, melatonin was separated on a Phenomenex Luna RP C18 column (150×4.6 mm i.d., 5 μm). The chromatographic system was isocratically operated with the following mobile phase: 75 mM sodium acetate, 28 % acetonitrile, pH 5.0, at a flow rate of 1 mL/min. One millilitre of plasma or standard was added to the top of a disposable SPE column (Oasis HLB 1 ml (30 mg) extraction cartridges; Waters Corp. Milford, MA, USA). Melatonin was eluted by adding 1 ml of dichloromethane to the column

(twice). The eluate was evaporated to dryness under nitrogen flow. The dried residue was then reconstituted in 100 μl of the mobile phase, and 20 μl of the solution was injected into the HPLC system. The areas of peak detected by fluorescent detector (Ex, 275 nm; Em, 345 nm) were used for quantification. Plasma melatonin levels were presented as picogrammes per millilitre.

Measurement of 6-Hydroxy Melatonin Sulphate

We used enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (GenWay Biotech Inc., San Diego, CA, USA) to determine urinary 6-HMS levels. The intra-assay coefficient of variation (CV %) was 5.2–12.2 %, and the inter-assay coefficient of variation was 5.1–14.9 %. After spiking, average spike recoveries ranged from 91 % to 122 % and, overall mean recovery of 105.8 % was found. We measured all samples in duplicate. Urine 6-HMS levels were presented as microgrammes per 24 h.

Statistical Analysis

All statistical analyses were performed by using the SPSS 13.0 (SPSS Inc., Chicago, IL, USA) statistical package. Distributions were evaluated by using one sample Kolmogorov–Smirnov test. Student *t* and Mann–Whitney *U* tests were used for testing differences between groups. The results were expressed as mean±standard deviation and median (minimum–maximum). The Spearman Rho correlation test was used to indicate relationships between variables. A probability level of <0.05 was considered statistically significant.

RESULTS

The results of biochemical measurements are shown in Table 1. In patients with TB, erythrocyte sedimentation rate, C-reactive protein levels and leukocyte counts were higher than those in control subjects.

Mean plasma melatonin levels were found to be significantly depressed in patients with TB compared to the control values [33.2 pg/mL (7.5–163.4 pg/mL) vs. 45.2 pg/mL (19.3–289.0 pg/mL), respectively, $p=0.037$] (Table 1). The mean concentration of plasma melatonin was 1.36-fold higher in the control group compared to patients with TB.

In patients with TB, mean urinary 6-HMS concentrations [6.5 μg/24 h (0.2–34.8 μg/24 h)] were also significantly lower than the levels in control patients [21.7 μg/24 h (3.9–99.8 μg/24 h)] $p<0.001$ (Table 1). We found urinary 6-HMS levels to be 3.38-fold higher in the control group compared to patients with TB.

In both TB patients and control subjects, plasma melatonin concentrations were not correlated with urine 6-HMS concentrations ($p>0.05$).

DISCUSSION

The major findings of our study are the significant reduction in both plasma melatonin and urine 6-HMS concentrations in patients with pulmonary TB compared to healthy controls. Based on the published literature and our knowledge, these results are the first data in patients with TB.

A decline in the immune function is associated with increased incidence of infectious diseases, cancer and degenerative diseases. In recent years, many studies

Table 1. Demographic and Biochemical Data in the Tuberculosis and Control Groups

	Groups		
Parameters	Tuberculosis ($n=31$)	Control ($n=31$)	*p*
Age (years)	22.9±3.8	23.6±3.4	NS[a]
Plasma melatonin (pg/mL)	33.2 (7.5–163.4)	45.2 (19.3–289.0)	0.037[b]
Urine 6-HMS (μg/24 h)	6.5 (0.2–34.8)	21.7 (3.9–99.8)	<0.001[b]
Leukocyte count ($\times 10^3/\mu L$)	7.91±1.98	6.26±1.25	<0.001[a]
ESR (mm/h)	30 (2–95)	2 (1–12)	<0.001[b]
CRP (mg/L)	28.2 (2.92–134.2)	3.21 (1–8.1)	<0.001[b]

All data were expressed as mean±standard deviation and median (min–max)
ESR erythrocyte sedimentation rate, *CRP* C-reactive protein, *NS* nonsignificant
[a] Student *t* test
[b] Mann–Whitney *U* test

have focused on the potential interaction between melatonin and the immune system [21]. Melatonin has significant immunomodulatory roles on the immune system. Interleukin (IL)-2 and interferon (IFN)-γ produced by Th1 cells are particularly effective in enhancing immune responses that involve macrophages and other phagocytes [22]. Maestroni *et al.* have shown that inhibition of melatonin synthesis causes a reduction of cellular and humoral responses in mice [23]. Jankovic *et al.* found that pinealectomized animals which lack a nighttime rise in melatonin cannot generate complete immune responses following the induction of experimental allergic encephalomyelitis or skin transplantation [24]. Moreover, various studies have confirmed that melatonin augments natural and acquired immunity in animals [25]. In another study, melatonin supplementation resulted in an increase in $CD4^+$ T cell activity and in the secretion of IL-2 [26]. Melatonin acts on the immune system by regulating cytokine production by immunocompetent cells. It enhances IL-2, IFN-γ and IL-6 production by cultured human mononuclear cells [27].

Santello *et al.* have proposed that melatonin may have an inhibitory effect on $CD4^+$ $CD25^+$ cell population and consequently allowing the proliferation of $CD4^+$ and $CD8^+$ [28]. These cells contribute to increased levels of IL-2 and IL-12, thus reducing the harmful pathologic effects of *Trypanosoma cruzi*. They have also demonstrated that the treatment of *T. cruzi* infected animals with melatonin stimulates an effective immune response. These beneficial effects of melatonin may be attributable to the upregulation of the Th1 response. The results of Santello *et al.* suggest that the early and continuous administration of melatonin may have beneficial immunomodulatory effects [28].

There are many studies documenting a relationship between melatonin and immune responsiveness [29]. Although there is some contrary information in the literature, most has established an immune enhancing effect of melatonin, especially when the immune system is compromised. Pinealectomy reduces immune responsiveness in the pigeon [30], suggesting that immune functions are at least partially regulated by physiological levels of melatonin. Moreover, the treatment with exogenous melatonin improves immune function [31]. Additionally, there is a relationship between melatonin supplementation and increased anti-inflammatory cytokine levels. Raghavendra *et al.* reported that treatment of antigen-primed mice with melatonin results in increased IL-10 and decreased tumor necrosis factor alpha (TNF-α) levels [32].

Many animal studies confirm that melatonin exerts a modulatory effect on the immune system. Melatonin has immuno-stimulatory properties [29] and may improve certain immune functions [33]. Melatonin enhances both natural and acquired immunity [16]. It has been reported that the administration of melatonin or its precursor tryptophan to immunized birds (especially in old animals) increased the humoral immune response and enhanced blood immunoglobulin levels [16].

Melatonin influences the immune system in many ways, both directly and indirectly. Melatonin is known to mediate seasonal changes in the immune system [34]. A few studies have analyzed the seasonal changes of TB, identifying peaks, both at the end of winter [35] and at the beginning of the summer season [36]. With regard to the seasonal variations in immune function it has been reported that melatonin plays an organizational role by modifying physiological status on a seasonal basis [37]. Therefore, the seasonality of TB infections may be associated with seasonal changes in the immune system caused by annual fluctuations in melatonin levels. This would be important for the response of an organism to disease or to therapeutic treatments.

In oxidative environments, MTb develops fairly well in macrophages [38]. Additionally, oxidative stress caused by infection leads to damage in the host tissues. Recently, some studies have shown that reduced oxidative stress protects host tissues from harmful effects by limiting inflammation associated with infection [39]. On the other hand, during the acute phase of TB infection multiple reactive oxygen and nitrogen species are produced as part of the cytokine inflammatory cascade and may be important in the killing of MTb. Melatonin and its metabolites are highly effective antioxidants and free radical scavengers, protecting against a number of radical species in both *in vivo* and *in vitro* models of oxidative stress [40, 41]. The combination of melatonin with traditional tuberculosis drugs may help to prevent cellular damage by the directing Th1 response.

It is well-known that the modulation of immune response may be caused by many disorders. Although the mechanisms of protection against TB have not been completely determined, cell-mediated immunity plays an important role in the control of MTb infection [42]. Immune modulation can present systematically as well as be localized it can be in the diseased organ.

The immunity in TB is cell-mediated, the intercellular interaction being mediated by cytokines which play an important role in determining the disease outcome. T lymphocytes are the predominant cells in TB document-

ing the role of cellular immune response. There are many studies investigating localized or systemic immune responses in TB [43]. It is accepted that, the Th1-type specific cellular immunity is responsible for protective immunity in TB while the Th2 response underlies the progressive course [44]. T lymphocytes and macrophages possess melatonin receptors, and they are also target cells for the immunomodulatory function of melatonin [45]. Cutolo *et al.* have shown that diurnal rhythms in cellular and humoral immune responses in humans are variable at different times of the day and are related to the immunomodulatory effects of melatonin [46]. Additionally, melatonin reportedly regulates gene expression of several immunomodulatory cytokines including TNF-α, transforming growth factor beta and stem cell factor by macrophages [47]. Therefore, melatonin may stimulate Th1 immune response in MTb infection and may protect Th1 immunity against acute MTb infection.

The role of melatonin during MTb infections is obviously not fully known. Suitable therapies are needed to prevent deaths due to TB. Therefore, in addition to the currently available drugs used to treat patients with TB, the addition of new medicines and products supporting the immune system such as melatonin may minimize deaths due to TB. Delay in diagnosing TB is an important worldwide problem. It seriously threatens public health. Plasma melatonin measurements are not routine tests in diagnostic laboratories. However, we believe that the measurement of plasma melatonin levels in patients with suspected TB infection could contribute to a better management of TB patients and may be important in terms of early treatment initiation. Thus, the immunomodulatory and antioxidative effects of melatonin suggest the possibility of its use as an additional therapeutic alternative to treatment of MTb infection.

Because of the administrative regulations, we were unable to collect blood samples at midnight. Although this is an important limitation for the current study, urinary 6-HMS results assisted us in the interpretation of the findings. This is the first study investigating plasma melatonin levels in patients with pulmonary TB. We believe that melatonin treatment in conjunction with traditional TB treatments may provide better cure results in patients with pulmonary TB. We also believe that melatonin treatment might result in a wide range of health benefits, improved quality of life and a reduction in the pathology of TB infections. Supplementation with melatonin may be considered as an adjunctive therapy to classic treatment of pulmonary TB especially during the acute phase of infection. Finally, further animal and human studies are required to clarify this presumption.

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