Melatonin improves inflammatory cytokine profiles in lung inflammation associated with sleep deprivation

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Received December 1, 2011; Accepted February 16, 2012

DOI: 10.3892/mmr.2012.814

Abstract. Sleep disturbance has become an endemic behavior in modern countries, and its prevalence has also increased. Even a subtle sleep deficiency is related to health problems. Particularly, patients with pulmonary disease often complain of insomnia. We recently showed that sleep deprivation (SD) exacerbates existing acute lung inflammation, and that melatonin treatment attenuates it via anti-apoptotic and anti-oxidant action. In order to reinforce our previous report, the present study was designed to evaluate pro-inflammatory mediators in acute lung inflammation in SD mice. In addition, we investigated the infiltration of inflammatory cells into the lungs. Twenty-five ICR mice were divided into 5 groups (n=5/group): control, SD, lipopolysaccharide (LPS), LPS + SD and LPS + SD + melatonin. The SD mice were deprived of sleep for 96 h in a multiplatform water bath. LPS (5 mg/kg) and melatonin (5 mg/kg) were administered on day 2. The mice were sacrificed on day 3, and serum and bronchoalveolar lavage (BAL) fluid were collected. The serum levels of inflammatory cytokines were increased in the LPS + SD group. Interleukin-6, tumor necrosis factor-α and interferon-γ levels were also increased in BAL fluid in the LPS + SD group. Melatonin reduced inflammatory mediators in the serum and BAL fluid. The accumulation of leukocytes in the LPS and LPS + SD mice was elevated, however, melatonin inhibited the recruitment of inflammatory cells ($p<0.05$). Lymphocytes in the BAL fluid of the LPS + SD group were increased, and macrophage levels were decreased; however, the increment was attenuated by melatonin administration ($p<0.05$). In conclusion, this study indicates that melatonin has a protective effect against lung inflammation associated with SD.

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Key words: acute lung inflammation, pro-inflammatory cytokines, sleep deprivation

Introduction

Proper amount of sleep is essential for maintaining health. Hence, sleep deprivation (SD) acts as an aggravating factor to patients with various diseases (1,2). Sleep deficiency is one of the most common complains in patients with respiratory diseases, and insomnia results in a significant deterioration in respiratory performance, even in a healthy person (3). It has been reported that SD is extremely common in the pulmonary intensive care unit (4). Sleep loss is related to a small but significant decline in respiratory function in chronic obstructive pulmonary disease (COPD) patients (5). When COPD patients were investigated, insomnia appeared to be related to health-related quality of life (6). The association between SD and lung inflammation has been studied. SD aggravates acute lung inflammation induced by lipopolysaccharide (LPS), including inflammatory cell infiltration, elevated apoptosis and membrane hyper-peroxidation (7). Even a subtle amount of sleep loss alters molecular processes that drive cellular immune activation and induce inflammatory cytokines (8).

LPS is a bacterial endotoxin and is widely used to induce acute lung inflammation (9). An important component of the LPS-induced inflammatory response of the lung is infiltration of polymorphonuclear (PMN) cells (10).

Melatonin, N-acetyl-5-methoxytryptamine, is the main neuroendocrine product of the pineal gland, and is found in other body fluids and tissues (11,12). Melatonin plays a regulatory role in the neuroimmuno-endocrine system (13). It has been speculated that melatonin and its metabolites directly scavenge reactive oxygen species as a potent anti-oxidant as well as acting as an anti-inflammatory agent (14).

In a previous study, we reported that melatonin protects the lung against inflammation associated with SD in mouse lung

tissue (7). In order to reinforce our previous report, the aim of the present study was to determine the pro-inflammatory cytokine levels in sera and alveoli. In addition, we investigated the accumulation of leukocytes in the lung with differential count in bronchoalveolar lavage (BAL) fluid.

The effect of melatonin on LPS-evoked pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ, was examined in serum and BAL fluid of SD mice. In addition, the accumulation and composition of leukocytes were measured with the Wright staining method and differential counting under a microscope for the evaluation of the effect of melatonin.

Materials and methods

Chemicals. Melatonin (N-acetyl-5-methoxytryptamine) and LPS (from the *Escherichia coli* serotype) were obtained from Sigma (St. Louis, MO, USA) and Fluka (Buchs, Switzerland), respectively. Melatonin and LPS were prepared as stock solutions of 1 mg/ml in ethanol and phosphate-buffered saline (PBS), respectively.

Animals and treatments. Seven-week-old male ICR mice (35-40 g) were obtained from Samtako Inc. (Kyunggido, Korea) and maintained in an air-conditioned room at room temperature (22-24˚C) and humidity (55-60%) with a 12-h light/dark cycle (lights on at 08:00 h), providing diet and water *ad libitum* during the entire experimental period. Body weights were measured every day. Special care was taken to avoid environmental stress before and during the experiments. All experiments were conducted in accordance with the animal care guidelines of the National Institutes of Health and Korean Academy of Medical Sciences. The mice were divided into 5 groups (n=5/group): control, SD only, LPS only, LPS + SD and LPS + SD + melatonin. Melatonin was intramuscularly administered at a dose of 5 mg/kg dissolved in 0.5 ml PBS shortly before intramuscular injection of LPS at a dose of 5 mg/kg. The control and SD mice were sensitized and challenged with PBS without drug treatment (Fig. 1).

SD. The SD mice were subjected to SD for a period of 3 days using a modified multiple platform water bath (15). The mice were placed in a filled water tank (56x40x18 cm) containing 18 circular platforms, 3 cm in diameter, in water up to within 1 cm of their upper surface. The control mice were placed on platforms of 8.5 cm in diameter. The mice could move inside the tank by jumping from one platform to another freely. When they reached the paradoxical phase of sleep, muscle atonia caused them to fall into the water and waked them up. The water in the tank was changed with clean water daily throughout the SD period.

Collection of serum and BAL fluid. Serum was obtained by centrifugation of blood at 800 x g at 4˚C for 15 min, and stored at -80˚C until analysis.

BAL fluid was collected by lavage of the lung via the trachea with 1 ml of PBS. After 5 lavages, ~0.7 ml of BAL fluid was recovered and centrifuged at 400 x g for 5 min at 4˚C. The supernatant was stored at -80˚C for the measurement of cytokines. The cells in the BAL fluid were resuspended in 100 µl of PBS for total cell and differential counts. After the measurement of the total cell number using a Neubauer hemocytometer, the cells suspended in PBS were applied to a slide by cytospin and stained with Wright stain solution. The percentage of each leukocyte was determined, and the absolute leukocyte count was calculated by multiplying each leukocyte percentage by the total cell count.

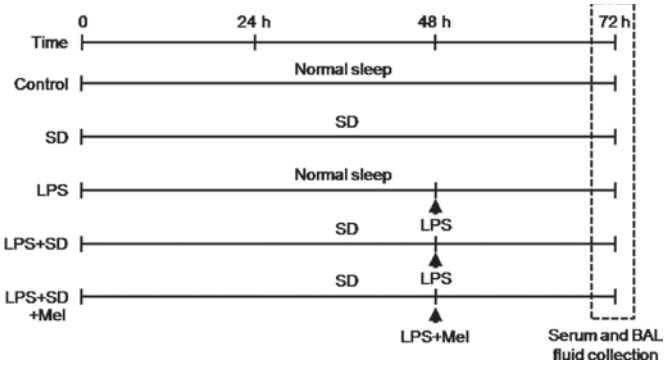

Figure 1. Schematic representation of the experimental design. Time-line illustrations of the sequence of events throughout the experiments show the groups of mice designated for the collection of serum and bronchoalveolar lavage (BAL) fluid (n=5/group). SD, sleep deprivation; LPS, lipopolysaccharide; Mel, melatonin.

Enzyme-linked immunosorbent assay (ELISA) of serum and BAL fluid. To determine the concentrations of cytokines (IL-1β, IL-6, TNF-α and IFN-γ) in the serum and BAL fluid, ELISA was performed using the Beadlyte Mouse Multi-Cytokine Detection System-2 kit (Upstate Biotechnology, Lake Placid, NA, USA) according to the manufacturer's instructions. Briefly, the multi-cytokine 2 standard was resuspended in assay buffer and then serially diluted from 2,500 to 15.6 pg/ml. Standard or sample (50 µl) was added to each well of a 96-well plate with 25 µl of the bead solution and was incubated overnight at 4˚C. The Beadlyte™ reporter solution was added to each well and incubated at room temperature for 1.5 h. Beadlyte streptavidin-phycoerythrin was diluted 1:25 in assay buffer, and was added to each well and incubated at room temperature for 30 min before the addition of the Beadlyte stop solution. The plate was then analyzed on the Luminex^{100} LabMAP™ system (Luminex Corp., Austin, TX, USA) using Masterplex QT software (MiraBio Inc., Alameda, CA, USA).

Statistical analysis. The data are expressed as the means ± SE of 11 samples analyzed in duplicate. Statistical analysis was performed using the one-way ANOVA followed by the Tukey test. The SPSS statistical software package (Version 14.0; SPSS Inc., Chicago, IL, USA) was used. A p-value of <0.05 was considered to indicate a statistically significant difference.

Results

We investigated the effects of melatonin on LPS and SD-evoked inflammatory cytokines in mouse serum and BAL fluid. In response to LPS, IL-1β, IL-6, TNF-α and IFN-γ in mouse serum were significantly increased after LPS administration (Fig. 2). The levels of IL-1β were increased in the LPS-treated

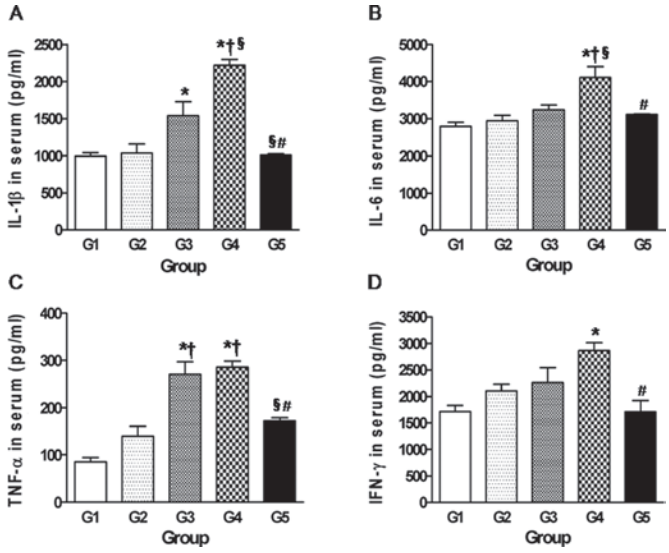

Figure 2. Effect of melatonin on the levels of cytokines in the serum. Blood was collected and serum was isolated. (A-D) Levels of IL-1β, IL-6, TNF-α and IFN-γ in the serum were analyzed by ELISA, as described in Materials and methods. Two independent experiments were performed. G1, control group; G2, SD group; G3, lipopolysaccharide (LPS) group; G4, LPS + SD group; G5, LPS + SD + melatonin group. Data represent the mean values ± SE for each group. *p<0.05 vs. G1; †p<0.05 vs. G2; §p<0.05 vs. G3; #p<0.05 vs. G4.

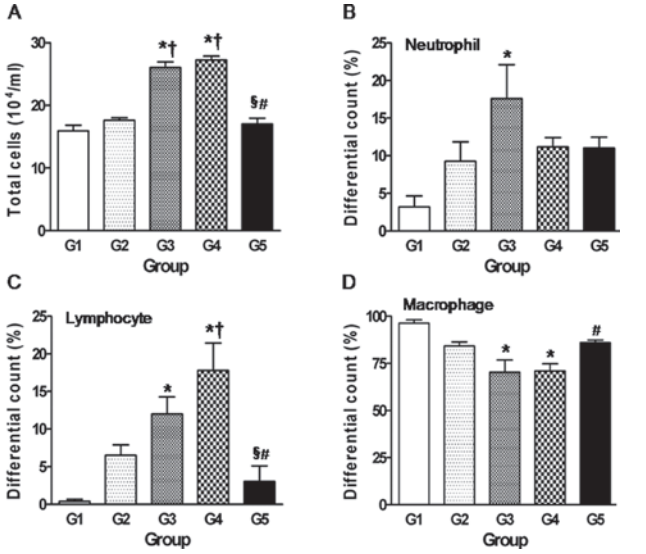

Figure 4. Effect of melatonin on the recruitment of inflammatory cells in bronchoalveolar lavage (BAL) fluid. Mice were divided into 5 groups: control (G1), SD (G2), LPS (G3), lipopolysaccharide (LPS) + SD (G4) and LPS + SD + melatonin (G5; each group, n=5). (A-D) The number of cells in the BAL fluid was totally and differentially counted after Wright's staining. Data represent the mean values ± SE for each group. *p<0.05 vs. G1; †p<0.05 vs. G2; §p<0.05 vs. G3; #p<0.05 vs. G4.

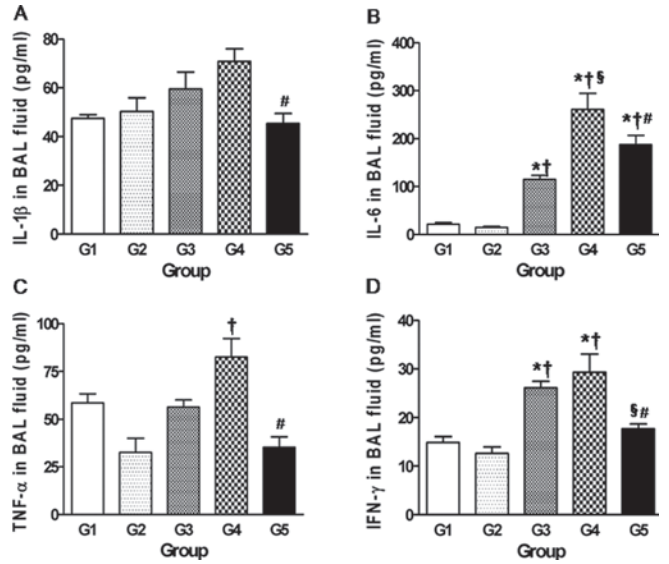

Figure 3. Effect of melatonin on the concentration of cytokines in bronchoalveolar lavage (BAL) fluid. BAL fluid was collected and the cells were eliminated. (A-D) The concentrations of IL-1β, IL-6, TNF-α and IFN-γ in BAL fluid were analyzed by ELISA. Two independent experiments were performed. Data represent the mean values ± SE for each group. G1, control group; G2, SD group; G3, lipopolysaccharide (LPS) group; G4, LPS + SD group; G5, LPS + SD + melatonin group. *p<0.05 vs. G1; †p<0.05 vs. G2; §p<0.05 vs. G3; #p<0.05 vs. G4.

mice [1,538.30±188.90 (mean ± SE)] as compared to the control mice, and this effect was more evident in LPS-treated SD animals (2,219.80±77.90). When mice were treated with melatonin, the levels of IL-1β were significantly decreased as compared to the LPS + SD group (1,006.70±18.90) (Fig. 2A). SD significantly increased the IL-6 levels in the LPS-treated mice (4,105.50±295.76). The serum levels of IL-6 were significantly attenuated after melatonin treatment (3,111.50±15.61) (Fig. 2B). Increased levels of TNF-α were observed in mice of both the LPS (271.33±25.46) and LPS + SD (286.00±12.66) groups. After melatonin treatment, a significant reduction in TNF-α levels in the serum was observed (172.00±6.93) (Fig. 2C). The serum levels of IFN-γ rose in the LPS + SD group (2,864.33±149.51), but were reduced by melatonin treatment (1,713.67±212.94) (Fig. 2D).

The levels of the pro-inflammatory cytokines, IL-6, TNF-α and IFN-γ, significantly increased in the BAL fluid of the LPS-treated mice (Fig. 3). No statistically significant difference was observed between the IL-1β levels of the control (47.50±1.44) and LPS-treated mice (59.50±6.98) (Fig. 3A). The administration of melatonin to the LPS + SD group significantly reduced the BAL fluid levels of IL-1β (45.33±4.10), IL-6 (187.50±19.56), TNF-α (35.33±5.49) and IFN-γ (17.67±1.01) (Fig. 3).

To examine the protective effect of melatonin against lung inflammation associated with SD, we evaluated the number of total leukocytes, including neutrophils, lymphocytes and macrophages, in the serum and BAL fluid. Melatonin significantly inhibited leukocytosis (17.05±0.93) (Fig. 4A). Differential leukocyte count showed that the lymphocyte number was increased in the LPS group (12.00±2.29) and the LPS + SD group (17.83±3.59). When mice were treated with melatonin, the number of lymphocytes was decreased (3.00±2.12) (Fig. 4). Macrophages in the LPS (70.40±6.41) and LPS + SD groups (71.00±3.79) were decreased, but melatonin treatment (86.00±1.47) increased the count of macrophages in the LPS + SD + melatonin group in comparison to the LPS + SD group (Fig. 4D).

Discussion

SD is reportedly common in patients with pulmonary diseases; it aggravates lung inflammation and vice versa (16). Melatonin has been reported to have a beneficial effect on inflammatory lung diseases (17). Herein, we found a protective effect of melatonin by measuring the levels of pro-inflammatory cytokines and leukocyte accumulation in the serum and BAL fluid of mice with lung inflammation induced by LPS and SD. Pro-inflammatory cytokines and the recruitment of inflammatory cells were elevated in the case of SD associated with lung inflammation, while melatonin decreased them significantly.

In order to investigate the effect of melatonin treatment, the inflammatory cytokines, IL-1β, IL-6, TNF-α and IFN-γ, were determined as molecular markers that indicate lung inflammation. Compared to the melatonin-treated LPS + SD mice, the concentrations of inflammatory cytokines were significantly increased in the serum of LPS + SD mice. When the serum levels of IL-1β, IL-6, TNF-α and IFN-γ in the LPS + SD and LPS + SD + melatonin mice were compared, melatonin treatment clearly attenuated the serum levels of inflammatory cytokines (Figs. 2 and 3). In particular, it was noted that animals treated with melatonin presented a significant reduction in inflammatory mediator levels in the BAL fluid of the LPS + SD group. Our data is consistent with other reports that melatonin reverses the elevation of pro-inflammatory cytokines in patients with respiratory distress syndrome (18).

It was prominent that leukocyte infiltration was significantly increased by LPS treatment, while melatonin treatment reduced the inflammatory cells in the mice BAL fluid. In this study, we showed that the pulmonary accumulation of lymphocytes was increased by LPS administration; the result of the LPS + SD group was the most remarkable (Fig. 4). On the other hand, melatonin treatment decreased the alveolar accumulation of lymphocytes. Our results are consistent with other reports demonstrating that the lymphocyte number was decreased by melatonin treatment (19). Human lymphocytes are a target for melatonin via binding sites in the regulation of immune function (20).

Moreover, melatonin administration increased the number of macrophages in the BAL fluid of the LPS + SD mice, and this result is consistent with other reports that melatonin treatment reduces acute lung injury induced by radiation therapy in rats (21). Our data demonstrate that pro-inflammatory cytokine levels are increased and that SD aggravates them in mice with lung inflammation induced by LPS. However, melatonin treatment significantly reduced the pro-inflammatory cytokine levels in the serum and BAL fluid. In conclusion, SD aggravates lung inflammation via the elevation of pro-inflammatory cytokines and the accumulation of leukocytes, and melatonin has a protective effect against lung inflammation associated with sleep loss. These results strongly reinforce our previous study and, thus, melatonin could be applicable as a therapeutic treatment in lung inflammation-associated SD.

Acknowledgements

The authors thank Ja-Hyun Kim and Ok-Soon Kim for their technical support. This study was supported by the Eulji University Research Fund (EJRG-07-001-12E04).

References

1. Friedman O, Bradley TD, Ruttanaumpawan P and Logan AG: Independent association of drug-resistant hypertension to reduced sleep duration and efficiency. Am J Hypertens 23: 174-179, 2010.
2. Sauvet F, Leftheriotis G, Gomez-Merino D, *et al*: Effect of acute sleep deprivation on vascular function in healthy subjects. J Appl Physiol 108: 68-75, 2010.
3. Cooper KR and Phillips BA: Effect of short-term sleep loss on breathing. J Appl Physiol 53: 855-858, 1982.
4. Shilo L, Dagan Y, Smorjik Y, *et al*: Effect of melatonin on sleep quality of COPD intensive care patients: a pilot study. Chronobiol Int 17: 71-76, 2000.
5. Phillips BA, Cooper KR and Burke TV: The effect of sleep loss on breathing in chronic obstructive pulmonary disease. Chest 91: 29-32, 1987.
6. van Manen JG, Bindels PJ, Dekker EW, *et al*: Added value of co-morbidity in predicting health-related quality of life in COPD patients. Respir Med 95: 496-504, 2001.
7. Lee YD, Kim JY, Lee KH, *et al*: Melatonin attenuates lipopolysaccharide-induced acute lung inflammation in sleep-deprived mice. J Pineal Res 46: 53-57, 2009.
8. Irwin MR, Wang M, Campomayor CO, *et al*: Sleep deprivation and activation of morning levels of cellular and genomic markers of inflammation. Arch Intern Med 166: 1756-1762, 2006.
9. Lopez A and Yong S: Injury versus inflammatory response in the lungs of rats intratracheally inoculated with bacterial lipopolysaccharide. Am J Vet Res 47: 1287-1292, 1986.
10. Sveen K: The importance of C5 and the role of the alternative complement pathway in leukocyte chemotaxis induced in vivo and in vitro by *Bacteroides fragilis* lipopolysaccharide. Acta Pathol Microbiol Scand B 86: 93-100, 1978.
11. Reiter RJ and Korkmaz A: Clinical aspects of melatonin. Saudi Med J 29: 1537-1547, 2008.
12. Shaw PF, Kennaway DJ and Seamark RF: Evidence of high concentrations of melatonin in lateral ventricular cerebrospinal fluid of sheep. J Pineal Res 6: 201-208, 1989.
13. Reiter RJ, Calvo JR, Karbownik M, *et al*: Melatonin and its relation to the immune system and inflammation. Ann NY Acad Sci 917: 376-386, 2000.
14. Mayo JC, Sainz RM, Tan DX, *et al*: Anti-inflammatory actions of melatonin and its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine(AFMK)andN1-acetyl-5-methoxykynuramine (AMK), in macrophages. J Neuroimmunol 165: 139-149, 2005.
15. van Hulzen ZJ and Coenen AM: Effects of paradoxical sleep deprivation on two-way avoidance acquisition. Physiol Behav 29: 581-587, 1982.
16. Amin R, Bean J, Burklow K, *et al*: The relationship between sleep disturbance and pulmonary function in stable pediatric cystic fibrosis patients. Chest 128: 1357-1363, 2005.
17. Martins E Jr, Ligeiro de Oliveira AP, Fialho de Araujo AM, *et al*: Melatonin modulates allergic lung inflammation. J Pineal Res 31: 363-369, 2001.
18. Gitto E, Reiter RJ, Amodio A, *et al*: Early indicators of chronic lung disease in preterm infants with respiratory distress syndrome and their inhibition by melatonin. J Pineal Res 36: 250-255, 2004.
19. Karimungi MG and Joshi BN: Diurnal sensitivity in melatonin-induced hematological changes in the male albino rat. Biol Signals 5: 283-290, 1996.
20. Lopez-Gonzalez MA, Calvo JR, Osuna C, *et al*: Interaction of melatonin with human lymphocytes: evidence for binding sites coupled to potentiation of cyclic AMP stimulated by vasoactive intestinal peptide and activation of cyclic GMP. J Pineal Res 12: 97-104, 1992.
21. Serin M, Gülbaş H, Gürses I, *et al*: The histopathological evaluation of the effectiveness of melatonin as a protectant against acute lung injury induced by radiation therapy in a rat model. Int J Radiat Biol 83: 187-193, 2007.