**Effect of Melatonin on Human Nighttime Endotoxaemia: Randomized, Double-blinded, Cross-over Study**

MAHDI ALAMILI¹, KLAUS BENDTZEN², JENS LYKKESFELDT³, JACOB ROSENBERG⁴ and ISMAIL GÖGENUR¹

¹Department of Surgery, Køge Hospital, University of Copenhagen, Køge, Denmark;  
²Institute for Inflammation Research, Department of Rheumatology, Rigs hospitalet, Copenhagen University Hospital, Copenhagen, Denmark;  
³Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark;  
⁴Department of Surgery, Herlev Hospital, University of Copenhagen, Herlev, Denmark

**Abstract.** Background: Endotoxaemia is widely used as an experimental model to study sepsis under controlled conditions. Nighttime endotoxaemia induces a more pronounced inflammatory stress response compared to daytime. Previously, we have shown that melatonin has antioxidative and anti-inflammatory effects in inflammatory response to daytime endotoxaemia. Herein, we examined the effect of melatonin in response to human nighttime endotoxaemia. Patients and Methods: Twelve healthy male volunteers were enrolled in a randomized, placebo-controlled, double-blinded cross-over trial. Subjects were induced by lipopolysaccharide (LPS) endotoxin 0.3 ng/kg body weight intravenously at 24:00. One hour prior to induction of endotoxaemia, an 8-h infusion of melatonin 100 mg or placebo was initiated. Blood samples were drawn before and 2, 4, 6 and 8 h after induction of endotoxaemia and plasma was tested for pro-inflammatory markers (tumor necrosis factor alpha, TNF-α, interleukin-1β, IL-1β, interleukin-1, IL-6, and YKL-40 ), anti-inflammatory markers (interleukin-1 receptor antagonist, IL-1Ra, interleukin-10, IL-10, soluble tumor necrosis factor receptors I and II, sTNF-RI and sTNF-RII ), marker for oxidative damage (malondialdehyde (MDA)) and antioxidative enzyme (ascorbic acid (AA) and dehydroascorbic acid (DHA)). Results: Compared to placebo, melatonin did not reduce plasma levels of any of pro- and anti-inflammatory markers and it also failed to influence levels of AA, DHA and MDA. Conclusion: Melatonin has no beneficial effect on inflammation and oxidative damage induced by nighttime endotoxaemia in contrast to daytime endotoxaemia.

Sepsis occurs in up to 240 cases per 100,000 patients each year in the USA and Europe (1, 2, 3). Some of these patients develop severe sepsis including septic shock and this development has been increasing in the past two decades (2, 4). One third of patients with sepsis and half of patients with severe sepsis require intensive care unit admission (4). The mortality is 16% for patients with sepsis increasing to 46% for patients with septic shock. The annual economic cost has been estimated to be 16.7 billion dollars in the USA (5).

Sepsis-initiated inflammatory processes and oxidative stress may cause multi-organ failure and several drugs targeting blood pressure, coagulation system, inflammatory cytokines and renal function have been investigated for therapeutic effects. Melatonin is a hormone secreted by the pineal gland with modulatory effect on the circadian rhythm of body functions. Melatonin has been also shown to exhibit a potent antioxidant and anti-inflammatory effect in sepsis, but mostly in animal models (6-16). Studies exploring the effect of melatonin on sepsis in adults are, therefore, needed (17, 18).

Human endotoxaemia, as a model of systemic inflammation, imitates the acute-phase response in sepsis (19). It provides a reproducible systemic inflammatory response with a defined onset and it is fully reversible. The acute phase response is induced by *E. coli* endotoxin lipopolysaccharide (LPS) administered intravenously to healthy volunteers. Using this model, we recently showed that the human endotoxic response exhibits a day-night variation with a more pronounced inflammatory response during nighttime endotoxaemia (20). We also demonstrated that melatonin had an anti-inflammatory and antioxidative effect on daytime endotoxaemia (21). In the present article, we wanted to examine the effect of melatonin in a nighttime acute phase response using the same human endotoxaemia model.
Discussion
In the present study we showed that during nighttime endotoxaemia, administration of 100 mg of melatonin did not reduce the plasma levels of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6), anti-inflammatory cytokines (IL-1Ra, IL-10) and soluble cytokine receptors (sTNF-RI, sTNF-RII) compared to with pre-values (Figures 1 and 2). There were no significant differences between the two groups in any of the inflammatory markers at any time-point (t=0, 2, 4, 6 and 8) (Figures 1 and 2).

Effect of melatonin on oxidative stress response. There were no significant differences between the two groups in general or in each time-points for MDA, AA and DHA (t=0, 2, 4, 6 and 8) (Figure 3).

Statistical analysis. All data were tested for normality by the Kolmogorov-Smirnov test. For testing differences in measurements between groups at certain time-points, the Wilcoxon Rank test was used. The two-way repeated measures of ANOVA was used to test for difference in measurements at different time-points between the melatonin group and the placebo group, where the two factors were time and treatments (melatonin versus placebo). All data were tested for normality using the Kolmogorov-Smirnov’s test after log-transformation, if necessary. The statistical analysis was made using the SPSS version 20 (SPSS, Chicago, IL, USA) and data are presented as mean±standard error of the mean (SEM). Results with p-values <0.05 was considered as statistically significant.

Results
The trial included 12 healthy men with a median age of 23 years (range=19-31) and a body mass index of 24 (range=21-26). Endotoxaemia was induced at the same time during both intervention days. The wash-out periods between the interventions had a median of 28 days (range=22-30).

Effect of melatonin on the inflammatory response. The plasma levels of all pro-inflammatory cytokines (IL-1β, TNF-α, IL-6), anti-inflammatory cytokines (IL-1Ra, IL-10), soluble cytokine receptors (sTNF-RI, sTNF-RII) and YKL-40 were significantly changed after onset of endotoxaemia compared to with pre-values (Figures 1 and 2). There were no significant differences between the two groups in any of the inflammatory markers at any time-point (t=0, 2, 4, 6 and 8) (Figures 1 and 2).

Effect of melatonin on oxidative stress response. There were no significant differences between the two groups in general or in each time-points for MDA, AA and DHA (t=0, 2, 4, 6 and 8) (Figure 3).

The patients were randomized to receive either melatonin or placebo on the first and second study days, respectively. Through a catheter in the cubital vein, melatonin and placebo were infused intravenously and initiated at 23:00, i.e. 1 hour prior to LPS administration, and continued for 8 hours. The melatonin (M5250, Sigma-Aldrich, St. Louis, MO, USA; 99 % purity by thin layer chromatography) was tested for sterility according to the European Pharmacopoeia requirements. The melatonin powder was dissolved in 2 ml ethanol (99%) and mixed with 1 l physiological saline. This melatonin powder solution was shown to be active in physicochemical examination involving the serum response element assay and high pressure liquid chromatography (data to be published elsewhere). Placebo was prepared by a mixture of 2 ml ethanol (99%) and 1 l physiological saline.

A week before each intervention day, the subjects underwent an adaptation period with standardized sleep (8 hours of sleep between 23:00 and 08:00), no caffeine intake, no alcohol intake and wearing sleep mask during sleep. At each intervention day, the volunteers were monitored with hourly measurements of blood pressure, temperature and heart rate. Blood samples were drawn from the subjects before initiation of melatonin/placebo infusions and additional blood samples were collected 2, 4, 6 and 8 hours after the injection of LPS. The blood samples were drawn in EDTA-tube and centrifuged at 3,000 rpm for 3 minutes; plasma was snap frozen at –80˚C until analysis.

The analyses of blood samples included the pro- and anti-inflammatory markers, tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-1 receptor antagonist (IL-1Ra), IL-6, IL-10, the soluble TNF receptors (sTNF-R) I and –II, and YKL-40; the oxidative stress markers malondialdehyde (MDA), ascorbic acid (AA) and dehydroascorbic acid (DHA) were tested as well.

The cytokines TNF-α, IL-1β, IL-1Ra, IL-6, IL-10 and sTNF-R I and -II were measured in a Luminex 100 IS analyzer (Luminex Corporation, Austin, TX, USA) using appropriate multiplex antibody bead kits (Invitrogen Corporation, Carlsbad, CA, USA). Data were analyzed using the StarStation version 2.0 software (Applied Cytometry Systems, Sheffield, UK). The lowest levels of detection were (pg/ml): TNF-α, 0.5; IL-1β, 1.0; IL-1Ra, 30.0; IL-6, 1.0; IL-10, 1.0; TNF-R1, 15.0; and TNF-R11, 15.0. Kit precisions were (CV%): TNF-α, 7.7; IL-1β, 4.4; IL-1Ra, 5.0; IL-6, 7.6; IL-10, 9.4; TNF-R1, 4.3; and TNF-R11, 7.9.

Blood sample procedures and methods for the determination of the oxidative markers, MDA, AA and DHA, were performed as described previously by using high pressure liquid chromatography (22, 23). The concentration of YKL-40 in the plasma was determined by a commercial enzyme-linked immunosorbent assay (Quidel, Santa Clara, CA, USA).

Ethics. The trial was monitored by the Good Clinical Practice (GCP) unit at Copenhagen University Hospital and was approved by the Danish Medicines Agency (EudraCT-nr 2009-017360-1), The
of the lipid membrane, lipid peroxidation, results in the formation of malondialdehyde (26, 27). The immuno-inflammatory response involves the formation of pro-inflammatory cytokines (19, 28) such as IL-1β, TNF-α and IL-6, and formation of anti-inflammatory cytokines (19, 29), for example IL-1Ra and IL-10. This model is controlled, fully reversible and widely used as an experimental model for sepsis in humans (19).

Melatonin has been shown to have potent antioxidative and anti-inflammatory effects (6-18, 30, 31). Numerous experimental studies have documented the ability of melatonin to de-toxify reactive oxygen species, induce antioxidative enzymes including superoxide dismutase, glutathione reductase and glutathione peroxidase (6, 8-10, 30, 31). Furthermore, the inhibitory effect of melatonin on the inflammatory response has been widely documented (7, 11-18).

Gitto et al. and Fulia et al. have studied the effect of melatonin on asphyxiated newborns (32), septic newborns (17), surgical neonates (33) and preterm infants with respiratory distress syndrome (34, 35). All these patient groups have been demonstrated to have increased oxidative stress response including oxidative damage and increased inflammatory response. Melatonin, given to these infants, significantly decreased the levels of free radicals (32), lipid peroxidation (MDA) (17, 32) and the pro-inflammatory cytokines TNF-α, IL-6 and IL-8 (33-35). Melatonin has also been studied on adult human patients (22, 23). In studies, where melatonin was given perioperatively to patients undergoing laparoscopic cholecystectomy (22) or abdominal aneurysm repair (23), no effect was observed on oxidative damage (MDA), ascorbic acid (AA) or inflammation (CRP).

The results of our study differ considerably from the above studies in that we could not demonstrate a nighttime
Effect of melatonin on a host concerning inflammatory and oxidative stress responses. We used a randomized, controlled, experimental model for acute phase response and imitating the initial processes of sepsis, whereas both Gitto and Fulia tested melatonin on patients with an on-going systemic inflammatory response. A strength of our study was that we standardized the onset of endotoxaemia. Thus, we could minimize the endogenous variation in the host response to endotoxaemia and also variations in pharmacodynamics and pharmacokinetics (36-38). We also administered melatonin intravenously thereby by-passing the liver metabolism of melatonin and preventing a variation as high as 37-fold in bioavailability after oral administration (39, 40). However, the metabolites of melatonin (6-hydroxymelatonin, N-acetyl-5-methoxykynuramine and N1-acetyl-N2-formyl-5-methoxykynuramine) have potent antioxidative and anti-inflammatory effects as well (39, 41-43). By giving melatonin intravenously, as we did, this hormone by-passes the liver metabolism explaining why no demonstrable strong effects of melatonin on oxidative damage and inflammation were obtained. Hypothetically, melatonin and its metabolites might exert a synergistic effect. This has not been clarified yet. A reason why an effect was shown in neonates (32-35) may be that neonates do not synthesize melatonin in the first three months after birth, resulting in potential different pharmacodynamic mechanisms. It has been documented that numerous extrapineal tissues and organs have the capacity to synthesize melatonin and secrete it locally (39). It has also been shown that melatonin levels in certain tissues are much higher than plasma melatonin levels (44-47). Therefore, cells, tissues and, indeed, the whole organism may be primed differently in neonates.

Another difference between our study and previous studies dealing with the effect of melatonin is that we standardized the administration time of melatonin. A day-night difference in the pharmacokinetics and pharmacodynamics of several drugs have been described (48, 49). Recently the effect of melatonin in animal models

![Figure 2](image-url)

**Figure 2.** Plasma levels of four anti-inflammatory cytokines and soluble cytokine receptors. The time point 0 indicates the administration of LPS endotoxin 0.3 ng/kg on 12 healthy men. Endotoxaemia with placebo is marked in red and melatonin in blue. Results from the two-way ANOVA: (i) interaction term (time*intervention) were not significant for any of the markers; (ii) between groups were not significant for any of the markers.
has been reported to vary during the day. This circadian variation has been shown on the effect of melatonin in tissue regeneration and the antitumor effect of melatonin (50,51). In animal endotoxic models, the effect of melatonin on inflammation (IL-6 and IL-10) and oxidative stress (ascorbic acid) showed no difference between day and night (52). In a recent study we examined the effect of melatonin in a human endotoxaemia model induced at daytime (at 12:00) (21) and demonstrated that markers of inflammation (IL-1β and YKL-40) and oxidative stress (ascorbic acid) were suppressed by infusion of 100 mg melatonin. In contrast, this effect was not present when identical melatonin dose was administered during nighttime endotoxaemia, as shown in this manuscript. Interestingly, when looking at percentage changes in mean values of each time-point between placebo and melatonin at nighttime endotoxaemia, we found that melatonin increased the levels of TNF-α (51% at t=4), IL-6 (66% at t=6), sTNF-R1 (11% at t=2) and sTNF-R2 (15% at t=6). None of these changes were significant. Neither did a time-shift in the curves between placebo and melatonin was found. This might indicate that the effect of melatonin is dependent on which time in the day it is given. Future studies have to consider time of administration when investigating the effects of melatonin.

In perspective, we could not demonstrate a beneficial effect of melatonin on nighttime endotoxaemia in healthy young men. The effect of melatonin in human models and in patients has to be further investigated taking into consideration the day-night variation of melatonin’s effect, the day-night variation of the systemic inflammatory response and the potential effect of the metabolites of melatonin when administered orally.

**Conflicts of Interest**

The Authors declare no conflicts of interest.

**Acknowledgements**

The project received grant from Aase og Ejnar Danielsens Fond, Familien Hede Nielsens Fond, The AP Møller Foundation for the advancement of medical science, snedkermester Sophus Jacobsen og hustru Astrid Jacobsens Fond.

**References**


46 Skinner DC and Malpau B: High melatonin concentrations in third ventricular cerebrospinal fluid are not due to Galen vein blood recirculating through the choroid plexus. Endocrinology 140: 4399-4405, 1999.

Received June 6, 2014
Revised July 21, 2014
Accepted July 22, 2014