# Circadian Variation in Endotoxaemia and Modulatory Effects of Melatonin

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**THIS THESIS IS BASED ON THE FOLLOWING PAPERS**

1. Alamili M, Klein M, Lykkesfeldt J, Rosenberg J, Gögenur I. Circadian variation in the response to experimental endotoxaemia and modulatory effects of exogenous melatonin. Chronobiol Int 2013;30:1174-80.
2. Alamili M, Bendtzen K, Lykkesfeldt J, Rosenberg J, Gögenur I. Pronounced inflammatory response to endotoxaemia during nighttime: a randomised cross-over trial. PLoS One 2014;9:e87413.
3. Alamili M, Bendtzen K, Lykkesfeldt J, Rosenberg J, Gögenur I. Melatonin suppresses markers of inflammation and oxidative damage in a human endotoxaemia model. J Crit Care 2014;29: 184.e9-184.e13.
4. Alamili M, Bendtzen K, Lykkesfeldt J, Rosenberg J, Gögenur I. Effect of melatonin on human nighttime endotoxaemia: randomized, double-blinded, cross-over study. In Vivo 2014;28:1057-63.

**INTRODUCTION**

A relationship between circadian rhythms and the pathophysiology of disease processes has been described intensively in the literature in the past decades [5-9]. On one hand, we have circadian disturbances that occur in relation to a specific disease process or surgical intervention. Both major and minor surgery have been shown to disturb the circadian rhythms of core body temperature, the secretion of the circadian hormone melatonin, cortisol levels in the blood, the autonomic nervous system, and the circadian distribution of sleep phases [10-19]. There is a "dose-response" relationship within this area, with the worst changes occurring in patients in the intensive care unit with severe sepsis [14]. On the other hand, endogenous circadian rhythms can affect the pathophysiology of diseases [19-21]. The exacerbation of asthma peaks during the night [18-20], acute myocardial infarction occurs more frequently in the early morning hours compared with the rest of the day [19-21, 25,26]. Sudden cardiac death, pulmonary thromboembolisms and stroke exhibits a circadian variation with higher concentration of these sometimes fatal events during the early morning hours [8]. This has also been demonstrated after surgery where there is a circadian difference in the distribution of post-operative cardiovascular events [4] as well as unexpected deaths occurring more frequently during the night compared with day and evening [27].

The immune system and immune response have also been suggested to have a function that depends on time of day [5,28,29]. Several studies have been reporting a circadian rhythm in the activity of the immune system and a circadian rhythm of inflammatory mediators in blood [30-34]. Only few studies have investigated the immune system response under a challenging condition [35-37]. One of the most demanding immune responses is sepsis. Sepsis is a systemic inflammatory response due to, for instance pathogens, e.g bacteria, virus and fungi. Patients with sepsis have high morbidity and mortality and the economic costs of the treatment of sepsis and the late complications of sepsis are increasing year by year [38]. Therefore, a greater scientific knowledge of the pathophysiology behind sepsis is needed. The treatment of sepsis is complex and although great attention has been directed on the treatment of this patient group, the mortality is still very high in patients with severe sepsis.

The interpretation of results originating from interventional studies on septic patients are challenging since this heterogeneous patient group has several factors that may influence the tested intervention. Experimental models for systemic inflammatory response are widely used in scientific studies on sepsis. One of these models is endotoxaemia, which is based on the administration of lipopolysaccharide endotoxin, eliciting a systemic acute phase response in biological organisms [39].

Melatonin is an endogenous endocrine hormone secreted by the pineal gland that maintains the circadian rhythm in both animals and humans [40]. The synthesis and secretion of melatonin are inhibited by daylight, and therefore the blood plasma levels of melatonin exhibits a circadian variation with a maximum peak during the dark period and a drop during the light period. It has been documented that melatonin also acts as a powerful antioxidant that reduces oxidative stress at many levels [36-38]. Furthermore, melatonin was demonstrated to inhibit the progression of the inflammatory response in several clinical situations with various degrees of inflammation [43-47]. Documentation in human studies is, however, limited [48-52].

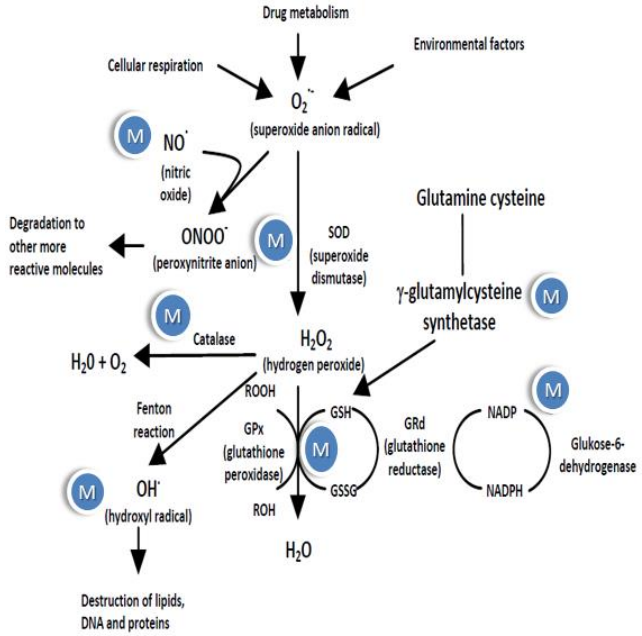


**Figure 1**
Oxidative stress and sites of action of melatonin (m). H2O=water, ROH=lipid alkoxid, ROOH=organic hydroperoxide, GSH=reduced glutathione, GSSG=oxidized glutathione, NADP=nicotinamide adenine dinucleotide phosphate, NADPH=reduced form of NADP.

In this PhD thesis it was investigated in animal and human experimental endotoxaemia models, whether there was a circadian difference in the inflammatory and oxidative stress response, and whether melatonin could modulate this response.

## BACKGROUND

### Circadian rhythms

A circadian rhythm is any biological process which displays an endogenous oscillation of about 24 hours. These rhythms are driven by a circadian clock and rhythms have been widely observed in plants, animals, fungi etc. Circadian is a term that origin from the Latin “circa”, meaning "around" and “diem” or “dies”, meaning "day". Although circadian rhythms are endogenous, they are entrained to the local environment by external cues called zeitgebers. One of the most powerfull zeitgeber is light.

The best known circadian rhythms in humans are sleeping, feeding patterns, bowel function, core body temperature, brain activity, hormone production and cell regeneration. The primary circadian clock in humans is located in the suprachiasmatic nucleus (SCN) located in the hypothalamus. The SCN receives information about illumination through the retina, which contains a photopigment called melanopsin and their signals follow a pathway called the retinohypothalamic tract, leading to the SCN. The SCN passes the information further to the pineal gland; in response to this, the pineal gland secretes the hormone melatonin into the blood stream. The secretion of melatonin peaks during the night/early morning hours, and ebbs during the day. The SCN regulates several body functions including the sleep/wake cycle, alertness, hormone secretion, temperature regulation, immune function, and the autonomic nervous system. Disturbances in the circadian rhythm are associated with increased mortality and morbidity [8].

Several medical conditions have been shown to disturb or diminish the circadian rhythm in the body. Patients with severe sepsis have impaired circadian rhythms of melatonin secretion [49]. Also surgical interventions impact the circadian rhythm of melatonin resulting in a shift of the peak of the plasma levels of melatonin from night to day [9,12,13,54]. Surgery also affects the sleep/wake cycle by reducing the number of REM sleep phases during the night [9-11,14].

It is also interesting that the pathophysiology of the diseases and symptoms of the diseases have been shown to exhibit a circadian rhythmicity [19-21]. It has been proven that there is a circadian peak in the presentation of symptoms and diseases at certain times of the day: blood pressure, stroke, acute myocardial infarction, sudden cardiac death, pulmonary thromboembolic events and stroke all exhibit a circadian variation [19-26].

Timing of medical treatment in coordination with the body clock may significantly increase efficacy and reduce drug toxicity. The administration of chemotherapeutic agents to patients with metastatic colorectal cancer at certain times of the day, instead of by continuous infusion, dramatically reduces toxicity and improves the oncostatic effect [55-59]. Treatment with angiotensin converting enzyme inhibitor may reduce nightly blood pressure and also benefit left ventricular remodeling, if dosed in a time-dependant manner [60].

### Sepsis

Sepsis is a potentially deadly medical condition that is characterized by a universal inflammatory state called a systemic inflammatory response syndrome and the presence of a known or suspected infection [61,62]. A development of the inflammatory response might be due to pathogens that originate from the blood, the urinary tract, the pulmonary system, the skin, or other tissues. Sepsis can gradually develop to more severe levels called severe sepsis [62], which is defined as sepsis with organ dysfunction, e.g. acute lung injury, acute respiratory distress syndrome, encephalopathy, heart failure, kidney dysfunction (oliguria, electrolyte abnormalities), coagulopathy, disruption in protein and metabolic functions, respiratory dysfunction, renal dysfunction, hepatic dysfunction, or haematological dysfunction. Patients can furthermore develop septic shock, which is defined as severe sepsis with refractory arterial hypotension or hypoperfusion despite intensive fluid treatment [62]. Sepsis can lead to multiple organ dysfunction syndrome and death [38]. Sepsis is a major challenge in the health system. Each year more than 500,000 cases of sepsis occur alone in the USA [63]. Approximately 1/3 of the patients with sepsis develop severe sepsis and half of these patients require intensive care unit treatment [38,64]. Approximately 20-35% of patients with severe sepsis and 40-60% of patients with septic shock die within 30 days [65]. Four percent of patients undergoing surgery develop sepsis, and 70 % of these develop severe sepsis [66].

The treatment of sepsis has been challenging through many years. Several drugs have been tested to improve outcome and morbidity. Steroids have failed to influence survival in severe sepsis and septic shock [67]. Recently, it has been shown that biological agents including endotoxin antibodies, cytokine inhibitors and receptor-antagonists could not improve survival of patients with sepsis and septic shock. Despite the wide range of available antibiotics, the mortality rate of Gram-negative bacteraemia complicated by septic shock is still approximately 50% [68].

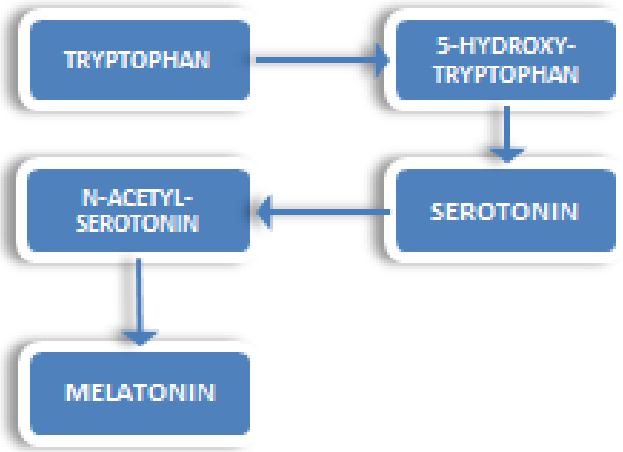


**Figure 2**
Melatonin synthesis from tryptophan.

The scientific work with sepsis implements problems and pitfalls. Septic patients are a very heterogeneous patient group; the treatment involves multiple drugs that may interact in unknown and unfavourable manners. Therefore, scientific work on sepsis is often performed on experimental sepsis models using cell cultures (in vitro and ex vivo), animal models or human volunteer models [39].

**Endotoxaemia**

The human endotoxaemia model is widely used and is a very suitable model for investigating sepsis under controlled conditions [39]. It is a reproducible systemic inflammatory response with a defined onset and the sepsis condition is fully reversible. The initial symptoms consisting of headaches, chills, and muscle pain occur at 60 minutes and peak at 90 minutes after endotoxin injection, followed by a gradual resolution over the next 2-3 hours [39].

The endotoxaemia activate the immune system and is induced by intravenous administration of lipopolysaccharide (LPS) endotoxin, mainly from Gram-negative bacteria. LPS is the major structural component of the outer wall of all Gram-negative bacteria and a potent activator of the immune system [69-72]. LPS consists of a polysaccharide region that is anchored in the outer bacterial membrane by a specific carbohydrate lipid moiety termed lipid A. Lipid A, also known as endotoxin, is responsible for the immunostimulatory activity of LPS. Lipid A is a glucosamine disaccharide linked to hydroxy fatty acids that are further substituted by nonhydroxylated fatty acids. The number of fatty acids is a major determinant of the immunogenicity of endotoxin [72]. The most active form of lipid A contains six fatty acyl groups and is found in pathogenic bacteria such as Escherichia coli and Salmonella species [73,74]. Underacylated lipid A structures, containing four or five fatty acids, induce markedly less host defense responses and can inhibit in a dose-dependent manner the strong endotoxic response triggered by hexa-acylated LPS. Such antagonists have been isolated from Rhodobacter sphaeroides and Porphyromonas gingivalis [75].

When LPS enters the body it is recognized by the toll-like receptor 4 (TLR-4) in complex with a transmembrane protein (CD14), the LPS binding protein (LPB) and myeloid differentiation protein 2 (MD-2). TLR-4 receptor is expressed on the surface of monocytes, macrophages, dendritic cells, intestinal epithelial cells, endothelial cells and in many other tissues. According to the current model, LPS is delivered to CD14 by LBP and transferred to MD-2 to form a monomeric endotoxin-MD-2 complex that binds and activates TLR4 [39,76]. It is the lipid A that binds to MD-2 and induces conformational changes, that trigger TLR4 oligomerization and signaling. The binding to the receptor initiates a downstream intracellular signaling pathway that finally results in the activation of NFκB, which in the end stimulates the transcription of genes coding for cytokines [76].

The cytokines can be divided into two types, pro-inflammatory and anti-inflammatory cytokines. The pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 promotes the development of inflammation by mediating important features of the acute phase response, such as the induction of fever, facilitating leukocyte migration, increasing tissue perfusion, and vascular permeability, all of which may facilitate the final eradication of the microorganism [77]. On the other hand the anti-inflammatory cytokines IL-10, IL-1Ra and IL-8 inhibit the progression of the inflammatory response. The anti-inflammatory response includes also soluble TNF-α receptors, that bind to TNF-α, thus attenuating the effects of this cytokine. The anti-inflammatory phase is central for the resolution of the immune response [78]. Another mediator of inflammation is YKL-40, which is produced by macrophages in the body [79,80]. YKL-40 has a function for both acute and chronic inflammatory processes [81]. Patients with Streptococcus pneumonia have elevated YKL-40 concentrations and this is also associated with severity as well as fatal outcome of the disease [82,83].

Endotoxaemia results in increased oxidative stress that may evolve to oxidative damage of tissues and cells [84-86] (figure 1). The oxidative stress is mediated by free radicals, which can be reactive oxygen species (ROS) or nitrogen reactive species (RNS) that have an unpaired electron in their valance orbital making them instable and highly reactive [85,87]. ROS include toxic products derived from oxygen including singlet oxygen ($^1O_2$), superoxide anion radical ( $O_2^{\bullet -}$ ), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($OH^\bullet$). RNS include nitric oxide ($NO^\bullet$), that is formed by the nitric oxide synthase (NOS), and peroxide nitric anion ($ONOO^-$). Oxygen is readily reduced to superoxide anion radical () as a result of normal cellular respiration that occurs in the mitochondria and under a variety of pathophysiological conditions, e.g. endotoxaemia and sepsis. The $O_2^{\bullet -}$ can couple to $NO^\bullet$ to generate $ONOO^-$ (can be degraded to more reactive species) or is dismutated by superoxide dismutase (SOD) to produce the oxygen metabolite ($H_2O_2$). The $H_2O_2$ is converted to $OH^\bullet$, which indiscriminiately destroys any molecule in the immediate vicinity. Because the relatively long half-life of $H_2O_2$ and its ability to penetrate cellular membranes, the $H_2O_2$ has the potential capability of spreading the damage associated with free radical generation. $H_2O_2$ can also be enzymatically converted to either $H_2O$ by the glutathione peroxidase (GPx) or catalse; or converted to the reactive molecule hypochlouros acid (HOCl) by the myeloperoxidase (MPO). The SOD, GPx and catalse are cellular enzymes that act as antioxidants by removing the reactive species. The GPx uses the substrate glutathione (GSS) to reduce $H_2O_2$ to $H_2O$. Once GSS is oxidized to GSSG it can be oxidized back to GSS by the glutathione reductase (GPd) [85,87]. Other antioxidants are non-enzymatic, such as ascorbic acid (vitamin C), tocopherol (vitamin E), β-caroten (vitamin A) and melatonin [40-43].

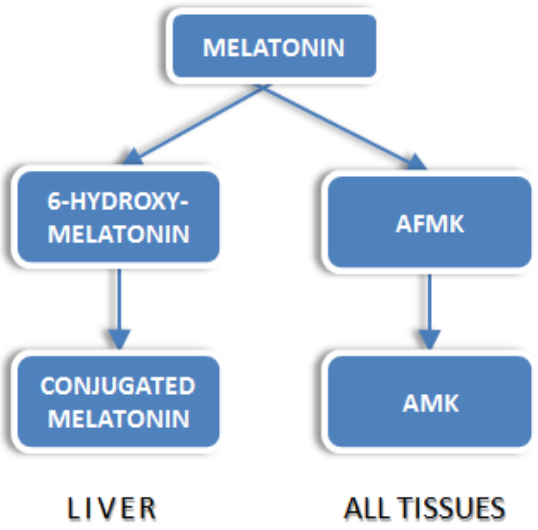


**Figure 3**
Melatonin metabolism in the liver and during interaction with free radicals in tissues. AFMK=N(1)-acetyl-N(2)-formyl-5-methoxykynuramine. AMK= (N(1)-acetyl-5-methoxykynuramine.

Oxidative stress represents an imbalance between reactive species and antioxidants in favour of the first, i.e. there are more reactive species than antioxidants when oxidative stress is apparent. This may lead to the damage of all components of the cell, including proteins, lipids, and DNA. This is called oxidative damage, and not all oxidative stress reactions may lead to oxidative damage. The oxidative stress is involved in the development of many diseases: cancer, Parkinson disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, schizophrenia, bipolar disorder, and sepsis [83]. In sepsis and endotoxaemia, the pro-inflammatory cytokines stimulate the immune cells to produce and secrete free radicals to eliminate pathogens [84,87]. During systemic inflammatory response a great amount of reactive species are secreted by the immune cells exerting a damaging effect on the host body tissues and cells [85,87]. The substrates produced by the interaction of oxidants with lipids (lipid peroxidation), proteins and DNA, are used as indicators for oxidative damage. Several methods have been developed to measure oxidative damage in the body, but only few of them are reliable. Malondialdehyde and isoprostanes are widely used to assess the lipid peroxidation [87], protein carbonyls for the protein oxidation and 8-oxogd (is an oxidized derivative of deoxyguanosine) for the oxidation of DNA.

**Methods used in the PhD studies for assessing the inflammatory response**

The endotoxaemia results in an increase in both pro-inflammatory cytokines and anti-inflammatory cytokines. The analyses of the levels of these cytokines in blood plasma can be done using different methods: enzyme-linked immunosorbent assay (ELISA), radioimmune assay and multiplex bead assay.

In study 1 we used an ELISA (Quantikine, R&D Systems Ltd, Abingdon, Oxon, UK) to determine the plasma levels of the cytokines IL-6 and IL-10 in rats. The blood was drawn in EDTA coated tubes, centrifuged, and plasma was stored at -80oC until analysis. Also the YKL-40 in study 2-4 was determined by ELISA method. The ELISA was based on a microplate that was coated with cytokine-specific antibodies, which captures the cytokines. The antibody-cytokine complex was then bound to an enzyme-linked polyclonal antibody specific for the cytokines, which in turn formed a colorimetric substrate that could be detected by a spectrophotometer [88].

In study 2-4 the analysis of the cytokines was based on multiplex bead assay (Luminex Corporation, Austin, Texas, USA). The pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and the anti-inflammatory mediators IL-1Ra, IL-10, sTNF-RI and sTNF-RII were determined in blood plasma, which were drawn in EDTA tubes, centrifuged and stored at -80oC until analysis. The multiplex bead assay is distinguished from procedures that measures one analyte at a time, measuring multiple cytokines simultaneously. Basically, the cytokines are captured onto spherical beads that are coated with cytokine specific antibodies. Next, a detector antibody binds to the bead-cytokine complex, and finally, a conjugated fluorescent protein binds, forming a solid phase, four-member sandwich, which is analysed with a Luminex detection system [85].

**Methods used in the PhD studies for assessing the oxidative damage and antioxidants**

The excess of reactive species can lead to damage of cell components and tissue, also called oxidative damage. Products produced by the interaction of oxidants with proteins, DNA and lipids (lipid peroxidation), are used as markers for oxidative damage. In the assessment of lipid peroxidation, the levels of malondialdehyde (MDA) and isoprostanes have been widely used as the golden standards [90]. In this thesis we chose to determine the levels of MDA in blood plasma. The analysis method of choice is the golden standard, and is based on high-performance liquid chromatography (HPLC) with fluorescence detection [87,91,92]. A pink fluorescence is formed when MDA reacts with thiobarbituric acid, which then is assessed by fluorimetry with excitation at 515 nm and emission at 553 nm. This method is considered to be the golden standard in clinical research settings dealing with oxidative damage and in research involving the test of pharmacological drugs targeting the oxidative damage [87,91].

Antioxidants can either be substrates or antioxidant enzymes, scavenging the reactive species, transforming them to harmless molecules. Many of these can be measured in blood samples and samples from different tissues. One of the most potent intra- and extracellular antioxidants is ascorbic acid (AA) [86,87]. When AA interacts with reactive species it is reduced to dehydroascorbic acid (DHA). AA scavenges superoxide, hyrdroxyl, peroxyl radicals, hypochlorite, and singlet oxygen. To avoid the redistribution of AA from plasma to erythrocytes and oxidation of AA, the blood samples for AA and DHA were drawn, handled and snap frozen within 5 min. The blood were collected in heparin tubes and centrifuged for 3 min. The obtained plasma was stabilized with 10% meta-phosphoric acid containing 2mM disodium EDTA to avoid the rapid oxidation of AA. The solution was centrifuged and the precipitate was removed. Finally, the samples were frozen and stored at -80oC until analysis. The determination of AA involved HPLC with coulometric detection, which also can measure the levels of total ascorbic acid (TAA). No method exists for the determination of DHA. Therefore, the levels of DHA in the plasma were calculated by the substraction of AA and TAA [93,94].

Another antioxidant measured in this thesis was superoxide dismutase (SOD), which plays a key antioxidant role [86,87]. SOD catalyzes the dismutation of superoxide ($O_2^-$) into hydrogen peroxide. Animals that lack SOD die shortly after birth and develop a wide range of pathologies. In study 1, a liver biopsy was

obtained after decapitating the rats. The sample were frozen immediately at -80oC until analysis. The pyrogallol method was used to quantify the activity of SOD in the liver tissue [95].

**The modulatory effect of melatonin in the acute immune response**

The synthesis of melatonin in the pineal gland begins with the hydroxylation and decarboxylation of tryptophan, which forms serotonin. Hereafter serotonin is N-acetylated and transformed to melatonin by hydroxyindole-O-methyltransferase (figure 2). Human melatonin production decreases as a person ages, and infant melatonin level becomes regular in about the 3rd month after birth [96].

***Antioxidative and anti-inflammatory effect of melatonin***

Besides its function as synchronizer of a biological clock, melatonin also exerts a powerful antioxidant activity [36-48] (figure 1). Melatonin has been shown to possess indirect and direct scavenging effect on free radicals such as $\mathbf{OH^{I}}$, and NO• [40-42,97]. The indirect effect involves an interaction between melatonin and receptors on the cell surface, while the direct effect is receptor independent [40-42]. The direct effect of melatonin can be targeted to the enzymes or to the reactive species. Melatonin stimulates the antioxidant enzymes SOD, GPx, GPd and CAT thereby increasing the elimination of and $H_2O_2$ and reducing the formation of the highly destructive $\mathbf{OH^{I}}$ [40]. The prooxidant enzymes NOS and MPO are inhibited by melatonin resulting in a decrease in the formation of HOCl and NO•. Beside this alternation in the activity of the enzymes in favour of a total antioxidant effect, melatonin also acts directly on the reactive species by scavenging. In scavenging, melatonin directly interacts with and NO• donating electrons to reduce the reactivity of the molecules. During this process melatonin is oxidized and generates c3OHM (cyclic 3-hydroxymelatonin) and AFMK (N(1)-acetyl-N(2)-formyl-5-methoxykynuramine) [40]. Both of these products are scavengers leading to the formation of another radical scavenger called AMK (N(1)-acetyl-5-methoxykynuramine). This cascade of generating new radical scavengers while reducing a reactive species, greatly increases the scavenging efficacy of melatonin [40].

Unlike other antioxidants melatonin does not undergo reduction cycling which is the ability of a molecule to undergo reduction and oxidation repeatedly. Reduction cycling may allow other antioxidants to act as pro-oxidants by promoting free radical formation. Melatonin, on the other hand, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant [40].

In the last two decades, melatonin has also been shown to have inhibiting effects on the development of inflammation [43-52]. Several studies have demonstrated that melatonin has a powerful anti-inflammatory effect in endotoxaemia [40]. This effect has been demonstrated on the blood plasma levels of the cytokines, decreasing the proinflammatory cytokines TNF-α, IL-1β, INF-γ, IL-6, IL-8 and IL-12 [98,99,100]; and increasing the anti-inflammatory cytokines IL-10 and IL-1Ra [99-101]. Melatonin also reduces superoxide production in the aorta and iNOS in the liver [98]. It also significantly decreases lung lipid peroxidation and counteracts the LPS induced increase of NO levels in lungs and liver [98,102-104]. Furthermore, melatonin reduced the production of free radicals in the mitochondria by inhibiting complexes 1 and 4 of the electron transport chain [104]. The development of apoptosis due to severe endotoxaemia was also significantly reduced by the administration of melatonin [99]. The effect of melatonin on LPS induced multi organ failure has also been evaluated. In animal models, melatonin counteracted development of kidney and metabolic dysfunction induced by LPS [102,105]. Melatonin prevents gastrointestinal disturbances in mice by reducing gastric empting of solid beads and altering the distribution of the beads throughout the gastrointestinal tract [106]. Melatonin reversed LPS induced intestinal motility disturbances and normalized the increased lipid peroxidation, iNOS expression and nitrite production in intestinal tissue [106]. In lungs, melatonin prevented the decrease in the PaO2, pulmonary oedema, elevated lung myeloperoxidase activity and lipid peroxidation after LPS [100].

***Pharmacokinetics of exogenous melatonin***

In laboratory rodents, the pharmacokinetics of melatonin including the absorption, bioavailability, half-life and clearance rate exhibit small individual variation [40]. The pharmacokinetics of melatonin in humans demonstrates a major individual variation [40,107-110]. Exogenous melatonin can either be administered orally or intravenously. Per oral melatonin is absorbed fully in the intestine and then transported to the liver where a first-pass metabolism takes place. During this process melatonin is hydroxylated and conjugated [111]. The bioavailability of melatonin is reported to differ up to 37 fold with great inter individual and gender variation [107]. The low bioavailability is attributed to its first pass effect through the liver probably due to the variation in the expression and activity of hepatic cytochrome CYP1A2 and CYP2C19 [112-114], and because melatonin may enter the bile and circulate in the hepato-enteric circulation [115]. In vitro studies suggest that the intestinal absorption of melatonin is not likely to be a significant barrier to the low oral bioavailability of melatonin [116]. Therefore, we intended to use intravenously administered melatonin in our human trials and subcutaneous melatonin in our rodent experiments.

When melatonin enters the blood stream it is distributed through the systemic circulation where it can be obtained by all tissue, including adipose tissues, because of its properties as a hydrophilic and lipophilic molecule. The metabolism of melatonin can either be in the liver by hydroxylation, where it is conjugated as 60-70 % sulphate and 20-30% glucuronide; or in other tissues by the direct interaction with free radicals [40,111] (figure 3). The metabolites of melatonin are excreted through the kidney with the major urinary metabolite being 6-sulphatoxymelatonin [117]. The plasma half-life is ½-1 hour for exogenous melatonin [40,108].

***Safety of melatonin***

In rats and mice, LD50 experiments indicate very low acute toxicity for melatonin [97,117]. Thus, the LD50 oral dose in Sprague-Dawley rats was over 3.2 g/kg body weight. In our rodent experiment we used 5 mg/kg body weight as a single dose administered intraperitoneally. The toxicity level can be measured by NOAEL, which is the level where no adverse effect is observed; and by LOAEL, which is the lowest level where adverse effect is observed. The NOEL and LOAEL for melatonin have been reported to be 100 mg/kg/day and 200 mg/kg/day, respectively [118].

In human studies regarding toxicity of melatonin, intravenously administered melatonin has been investigated with no side effects. In healthy subjects both 0.25 mg/kg body weight and 1.25 mg/kg body weight given intravenously did not show undesirable effects [115]. In a series of studies in newborns, melatonin

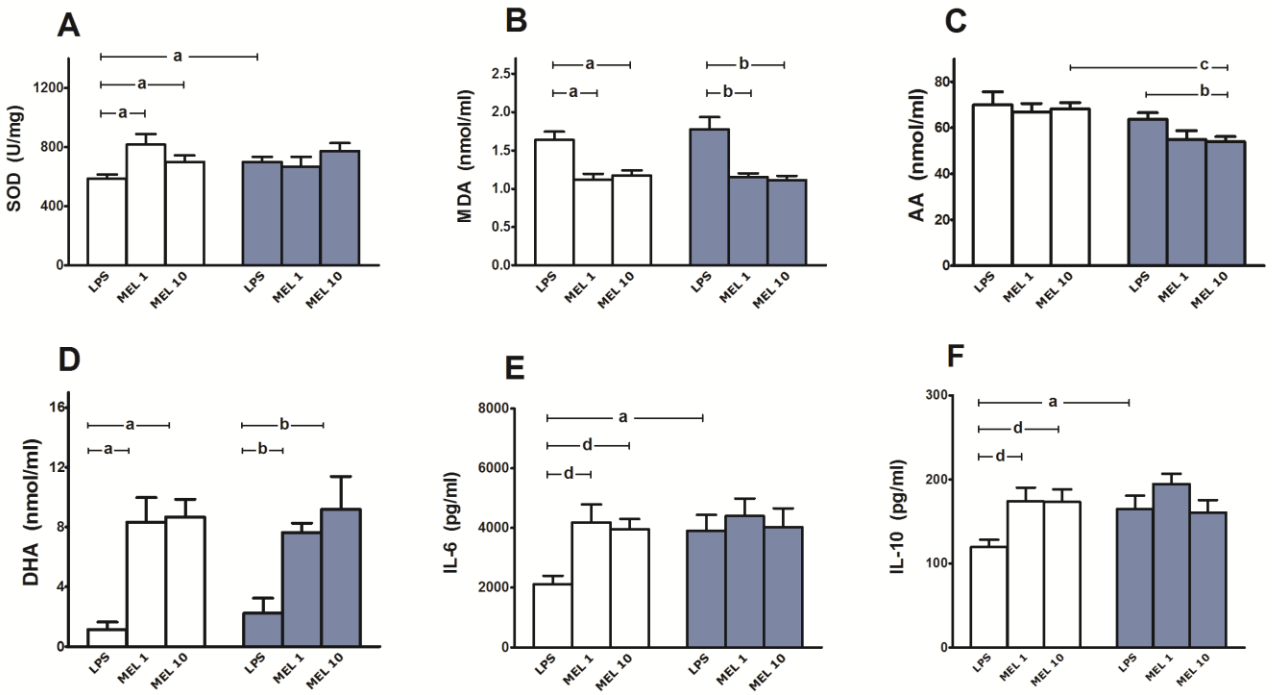


**Figure 4**
Plasma levels of oxidative and inflammatory markers. The levels are calculated as mean difference between samples collected just before the LPS 5 mg/kg injection and 5 hours after the onset of endotoxaemia. Daytime (white box), nighttime (blue box). MDA (malondialdehyde), SOD (superoxide dismutase), AA (ascorbic acid), DHA (dehydrogenized ascorbate), IL-6 (interleukin-6), IL-10 (interleukin-10). (a) P<0.05 vs. LPS daytime. (b) P<0.05 vs. LPS nighttime. (c) P<0.05 vs. melatonin 10 mg daytime. (d) P<0.01 vs. LPS daytime.

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was not associated with any side effects when administered orally with doses up to 10 mg/kg body weight [52,120]. In a recent study, 40 mg of intravenous infusion of melatonin was given to patients undergoing major vascular surgery. No adverse effects were reported in this trial [121].

**STATISTICAL CONSIDERATIONS**

Data in all studies are reported as mean and standard error of the mean (SE). All data were tested for normality using the Kolmogorov-Smirnov test. Data that were not normally distributed were log-transformated to become normally distributed. The transformation to normality was necessary for the cytokines. The paired Student T-test was used in the rodent study (study 1) except for the SOD where the unpaired Student T-test was used. In studies 2-4 we applied the Wilcoxon signed-rank test for the comparison between groups for certain time-points for the same persons in the cross-over study design. The two-way analysis of variance (ANOVA) was used to test for significance between two groups for the entire series of time-point measurements. For all studies, a P-value less than 0.05 was considered statistically significant. The SPSS 18.0 (IBM, Chicago, Illinois, USA) was used for the analyses.

**ETHICAL CONSIDERATIONS**

For study 1 approval from the Danish Experimental Animal Inspectorate was obtained (Journal-nr. 2009/561-1754). The rats were handled carefully and during blood sample drawing and decapitation animals were anaesthetized with isoflurane inhalation. Studies 2-4 were approved by the Regional Committee on Biomedical Research Ethics (H-2-2010-010), The Danish Data Protection Agency, and the Danish Medicine Agency (EudraCT-no. 2009-017360-1). All study subjects gave written informed consent before enrolment in the study. The Good Clinical Practice (GCP) Unit at Copenhagen University monitored the study. The human trial was registered at www.clinicaltrials.gov (NCT01087359).

**EXPERIMENTAL STUDIES**

**Study 1**

***Aim and design***

The aim of this study was to investigate whether there was a difference in the acute phase response due to endotoxaemia. Furthermore, the effect of melatonin on inflammation and oxidative stress was studied. This effect was investigated both at night and during daytime [1]. We included 60 rats (Sprague-Dawley) that were divided in 6 different groups, and all animals were injected with LPS endotoxin 5 mg/kg intraperitoneally. Animals in group 1 received endotoxin at daytime (zeitgeber time ZT02), while animals in group 2 were injected with endotoxin at night time (zeitgeber time ZT14). In groups 3 and 4 the animals received melatonin 1 mg/kg intraperitoneally at daytime or night time, respectively. Finally animals in group 5 and 6 received melatonin 10 mg/kg i.p. at daytime or night time respectively. Blood samples were drawn from the retro-orbital plexus before and 5 hours after the injections of LPS with or without melatonin.

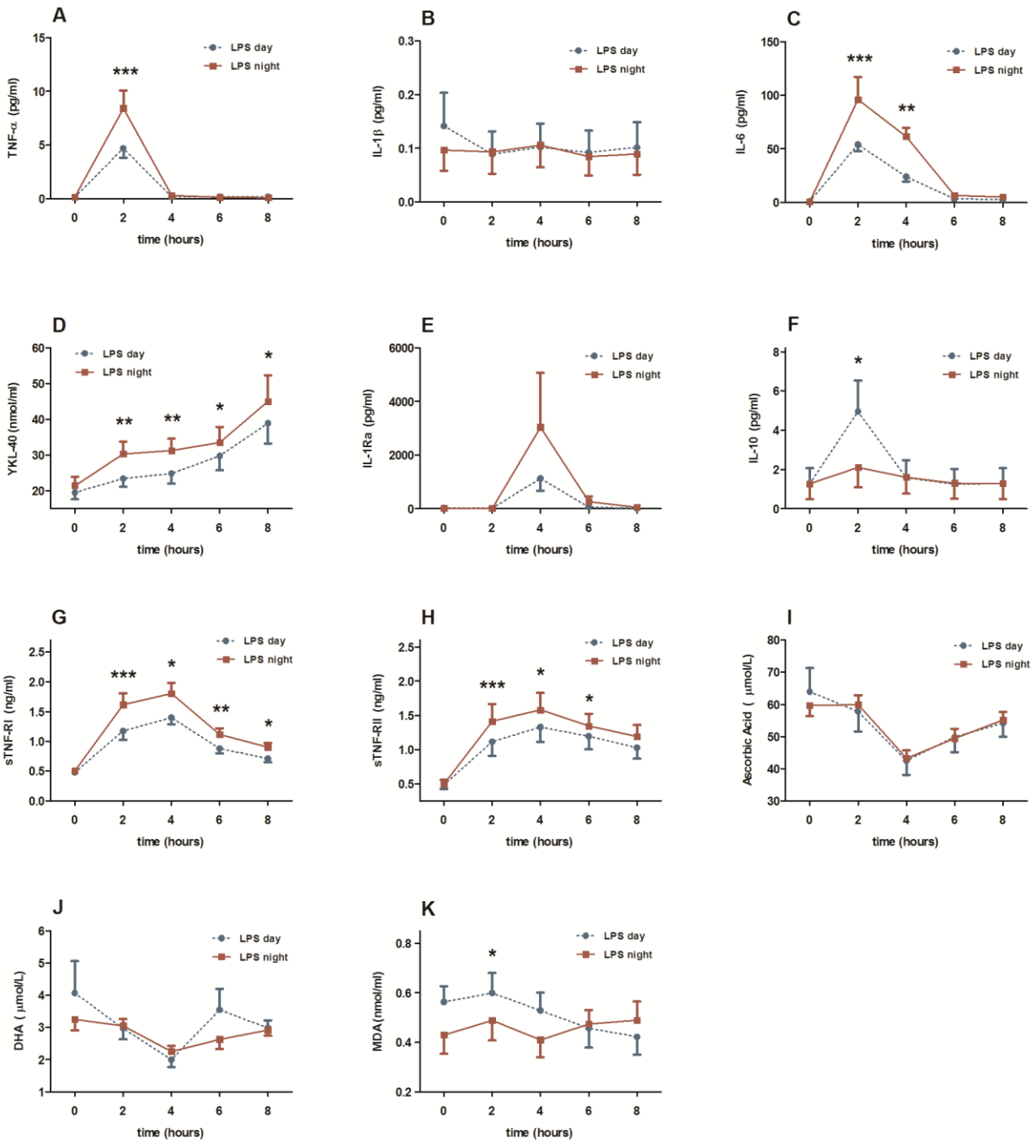


**Figure 5**

Plasma levels of inflammation and oxidation markers. The time point 0 indicates the administration of E. coli endotoxin (LPS). The endotoxaemia was induced at day time (blue curve) and night time (red curve). Results from the two-way ANOVA: (1) interaction term (time*day) were significant for IL-10 ($P < 0.001$) and MDA ($P < 0.05$), (2) between groups analyses were significant for IL-6 ($P < 0.0001$), YKL-40 ($P < 0.001$), IL-1Ra ($P < 0.05$), sTNF-RI ($P < 0.000000001$) and sTNF-RII ($P < 0.000001$) and MDA ($P < 0.05$). *) P-value < 0.05 calculated by Wilcoxon-Rank test, **) P-value < 0.01 calculated by Wilcoxon-Rank test, ***) P-value < 0.001 calculated by Wilcoxon-Rank test.

Hereafter, the animals were decapitated and immediately hereafter, the abdominal wall was opened and a liver biopsy was obtained. The blood samples were then analysed for oxidative markers (MDA, AA and DHA) and inflammatory markers (IL-6 and IL-10), while the liver biopsy was used for the determination of SOD.

***Results***

The endotoxaemia exhibited a day-night difference, where SOD ($P < 0.05$), IL-6 ($P < 0.01$) and IL-10 ($P < 0.05$) showed significantly higher levels during the nighttime compared with daytime (figure 4). Melatonin 1 and 10 mg/kg administered at daytime reduced the level of MDA ($P < 0.01$), and increased levels of DHA ($P < 0.001$) and SOD ($P < 0.05$). Furthermore, IL-6 and IL-10 ($P < 0.01$) were significantly increased. At night time melatonin 1 and 10 mg/kg reduced the levels of MDA ($P < 0.01$) and increased the levels of DHA ($P < 0.05$). Ascorbic acid was only reduced by melatonin 10 mg/kg ($P < 0.05$). There were no differences in the effect on oxidative and inflammatory markers in the low- and high-dose groups. A circadian variation in the effect of melatonin on endotoxaemia was only seen with melatonin 10 mg/kg on ascorbic acid ($P < 0.05$), where higher levels during daytime were found compared with night time.

***Limitations***

A limitation is the simultaneous injection of melatonin and LPS endotoxin intraperitoneally. It is known that LPS endotoxin results in a chemical irritation in the peritoneum thereby altering the permeability of the peritoneum, which might reduce the absorption of melatonin given to the animals. Although the inflammatory and oxidative markers included in this study are reliable and strong indicators for the level of inflammatory response and oxidative damage, other cytokines such as TNF-α and IL-1B could be included. These cytokines are crucial and have more potent pro-inflammatory effects than IL-6. We did not measure end-products of the degradation of proteins during the oxidative damage. Ascorbic acid is known to be produced by the liver in rats. Therefore, the amount of ascorbic acid that might be used in reducing the free radicals could be substituted by newly synthesized ascorbic acid from the liver.

**Study 2**

***Aim and design***

The aim of this study was to investigate whether the circadian variation in the endotoxaemic response that was seen in study 1 in the animal model could be reproduced in humans [2]. A randomized, cross over trial was initiated with 12 healthy young men who received LPS endotoxin 0.3 mg/kg at two different times during the day. In one day, the endotoxaemia was induced at daytime (12:00 a.m.) and at nighttime (12:00 p.m.) on the second day. Before each study day, subjects were acclimatized for a week with standardized sleep pattern and no intake of caffeine or alcoholic drinks. A wash out period for more than three weeks was inserted between the two study days to eliminate the endotoxin tolerance. In each study day, the subjects received endotoxin and blood samples were drawn before and 2, 4, 6 and 8 hours after the onset of endotoxaemia. The blood samples were then stored and later analysed for inflammatory and oxidative markers. The inflammatory markers included the pro-inflammatory cytokines TNF-a, IL-1B, IL-6 and the macrophages mediator YKL-40, and the anti-inflammatory cytokines IL-1RA, IL-10 and the soluble TNF-α receptor antagonists sTNF-RI, sTNF-RII. The end-product of the lipid peroxidation, malondialdehyde (MDA), and the antioxidant ascorbic acid were also measured, while dehydroascorbic acid was calculated.

***Results***

A day-night difference was seen in the inflammatory and the oxidative stress response. The pro-inflammatory cytokines TNF-α and IL-6, but not IL-1B, showed higher levels at night compared to day (figure 5). Also the anti-inflammatory cytokines IL-1Ra, and the soluble TNF-receptors, were higher at night (figure 6). The levels of MDA and the anti-inflammatory cytokine IL-10 showed higher levels during daytime endotoxaemia (figure 6). No differences were seen in the levels of ascorbic acid and dehydroascorbic acid between day and night time (figure 6).

***Limitations***

The dose of LPS endotoxin used in this trial induces a moderate acute phase response. The trial was an experimental study with endotoxaemia, which mimics the initial phase of sepsis, and therefore cannot be used to study the clinical progression in sepsis beyond the acute phase response. Finally, sepsis can be initiated by different pathogen-associated molecule patterns other than LPS endotoxin, i.e. peptidoglycan, which may initiate sepsis through other cellular pathways resulting in a different acute phase response.

**Study 3**

***Aim and design***

The aim of this study was to investigate whether melatonin had anti-inflammatory and/or anti-oxidative effects in a human endotoxaemia model [3]. We standardized the onset of endotoxaemia at daytime based on the day-night differences in the acute phase response induced by endotoxaemia, which we showed previously in both animal [1] and human models [2]. In the animal study, we showed that there was a day-night difference in the effect of melatonin on inflammation and antioxidant capacity. Therefore, the administration of melatonin was also standardized in this trial. Twelve healthy young men were included in a randomized, cross-over, double-blinded, placebo-controlled trial that consisted of two study days where the subjects received LPS endotoxin, and in one day they received melatonin 100 mg infusion intravenously for 8 hours and in the other day they received placebo infusion intravenously for 8 hours. The endotoxaemia was induced at 12 a.m. in both days and the infusion of melatonin/placebo was started at 11 a.m. and was continued for 8 hours. Between the two study days a washout period was included to eliminate the endotoxin tolerance. Blood samples were drawn before the onset of endotoxaemia and 2,4,6,8 hours after the onset of endotoxaemia. The blood was then analysed for pro-inflammatory mediators (TNF-a, IL-1B, IL-6, and YKL-40), anti-inflammatory mediators (IL-1Ra, IL-10, sTNF-RI, sTNF-RII), lipid peroxidation (MDA), and antioxidants (AA, DHA).

***Results***

Melatonin reduced significantly the plasma levels of the strong pro-inflammatory cytokine IL-1β ($P < 0.01$) but not TNF-α and IL-6. None of the anti-inflammatory cytokines (IL-1Ra, IL-10) and the soluble cytokine receptors (sTNF-RI, sTNF-RII) were reduced by melatonin (figure 7). Furthermore, the pro-inflammatory neutrophil mediator YKL-40 was significantly reduced by melatonin ($P < 0.05$, figure 8). Melatonin reduced the levels of AA ($P < 0.05$) but not DHA and MDA (figure 7).

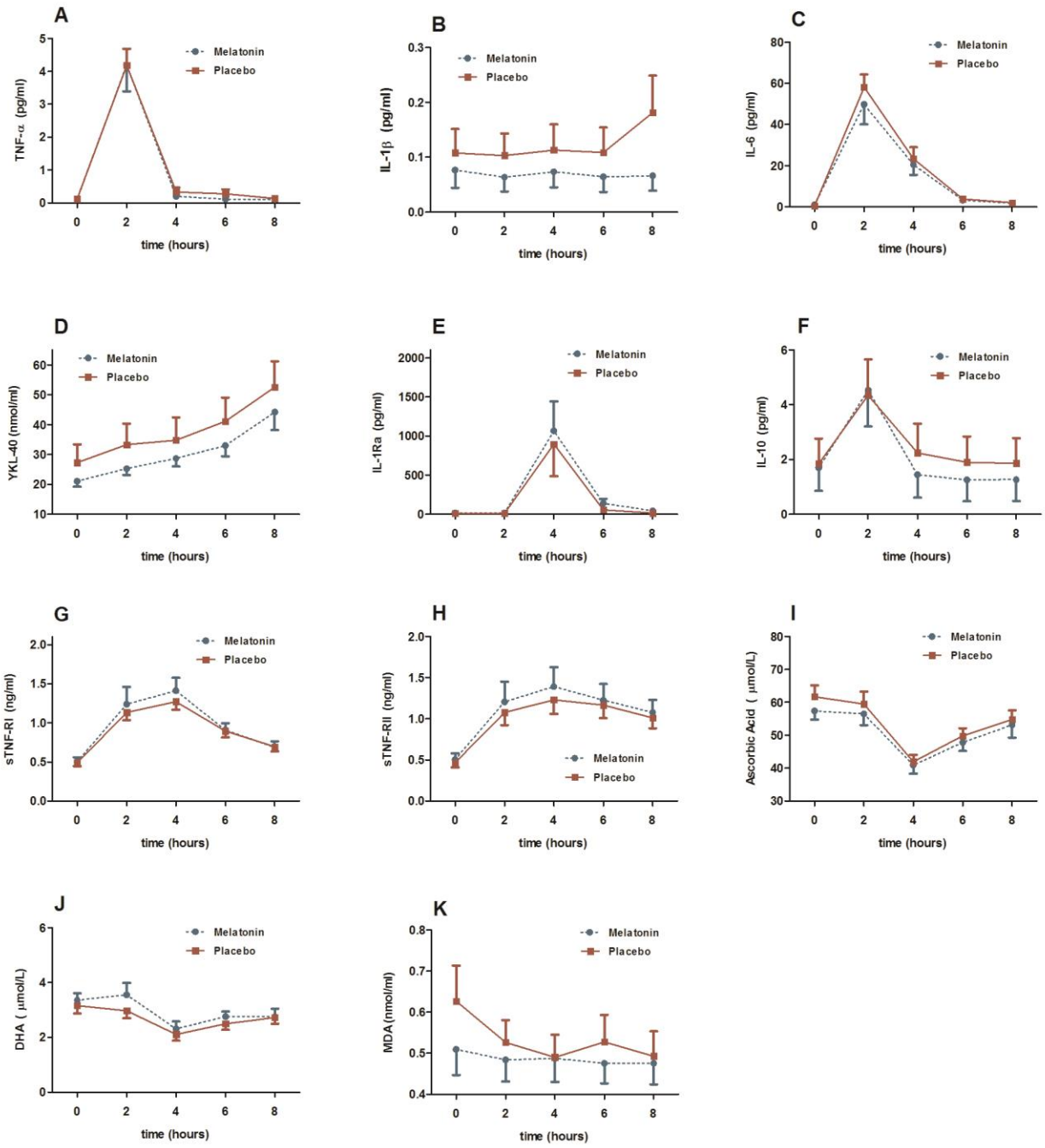


**Figure 6**

Effect of melatonin in daytime endotoxaemia. Plasma levels of inflammation and oxidation markers. The time point 0 indicates the administration of E. coli endotoxin (LPS). Plasma levels of three pro-inflammatory markers and YKL-40. The time point 0 indicates the administration of E. coli endotoxin. The endotoxaemia was induced at 12 a.m. and melatonin (blue curve) or placebo (red curve) was given before the onset of the endotoxaemia. Results from the two-way ANOVA: (1) interaction term (time*intervention) was not significant for any of the markers, (2) between groups was significant for IL-1B ($P < 0.01$), YKL-40 ($P < 0.05$) and AA ($P < 0.05$).

***Limitations***

The small amount of subjects included in the trial may have resulted in a type-II error. Furthermore, because the data cannot be extrapolated to sepsis, since the endotoxaemia induces an acute phase response that is time-limited and does not evolve to further phases of sepsis, it is unknown whether melatonin will have a beneficial effect on clinical sepsis. The dose of melatonin (approximately 1.25 mg/kg b.w.) used in this trial could be increased to higher levels, when compared to other human studies where melatonin 20 mg ( > 5 mg/kg body weight) was given to newborns and showing a beneficial effect on oxidative damage and inflammatory response. The administration pathway of melatonin was intravenous in this trial, thereby bypassing the liver metabolism of melatonin. Finally, we investigated the prophylactic effect of melatonin rather than the therapeutic, meaning that the patient has to be loaded with melatonin before the initiation of sepsis if our results should be fully applicable to the clinical situation.

**Study 4**

***Aim and design***

The aim of this trial was to investigate whether melatonin had an effect on endotoxaemia initiated during the night [4]. In previous studies we showed that endotoxaemia exhibited a day-night difference both in animal [1] and in human models [2]; and we demonstrated that melatonin had a beneficial effect on inflammation and oxidation in animal models [1] and to a certain extent also in human models [3]. Under normal conditions endogenous levels of melatonin peaks during the night, but this rhythmicity has been shown to be impaired under septic conditions. Therefore, we tested whether melatonin would have an effect during nocturnal endotoxaemia.

A study with the same setup as Study 3 was initiated with 12 healthy young men, where endotoxaemia was induced at 12 p.m. with intravenous infusion of melatonin 100 mg or placebo for 8 hours initiated at 11 p.m. The trial was a randomized, cross-over, double-blinded, experimental study. Before the onset of the endotoxaemia and 2, 4, 6, 8 hours after the onset, blood samples were drawn for analyses of the pro-inflammatory mediators (TNF-α, IL-1β, IL-6), anti-inflammatory mediators (IL-1Ra, IL-10, sTNF-RI, sTNF-RII), lipid peroxidation (MDA), and antioxidants (AA, DHA).

***Results***

Melatonin compared to placebo did not show any significant effects on pro-inflammatory markers, anti-inflammatory markers, oxidative damage or anti-oxidants (figure 8).

***Limitations***

Many of the limitations in this study are the same as in study 3. In addition to these, because the levels of endogenous melatonin are much higher during the dark period, the body may be in a saturated phase regarding its capacity to be effected by exogenous melatonin. The role of endogenous levels of melatonin during the night should therefore probably be examined further.

**DISCUSSION**

**Circadian variation in endotoxaemia**

***Inflammation***

We demonstrated that the acute inflammatory response due to LPS endotoxin exhibited a profound day-night variation. Both *proinflammatory* mediators and anti-inflammatory mediators developed higher plasma levels during night time compared to daytime, except for the anti-inflammatory cytokine IL-10 that showed higher levels during daytime in human models. Although rats and humans have opposite activity rhythms, with rats being active at night and humans are active in daytime, the inflammatory responses due to endotoxaemia showed higher levels during night time in both human and rats. This might indicate that the endogenous activity rhythm and the sleep-wake cycle in the body is not important for the day-night variation, but rather the endogenous rhythm is controlled by certain external cues, such as light and the endogenous rhythm of melatonin, as indicated by previous studies [20].

In 1960, Halberg et al., was the first to investigate the circadian variation in the inflammatory response to endotoxaemia in animal models [35]. Halberg observed that the lethality of E. Coli endotoxin varied approximately 10-fold depending upon when in the circadian rhythm the endotoxin exposure occurred. Mice were injected with 5 mg/kg E. Coli LPS intraperitoneally at four-hour intervals. The mortality differed dramatically peaking in the late day hours and with a minimum at midnight. Later in 1994, Hrushesky et al. demonstrated that the lethality effect of TNF-α administration varied 9-fold, depending upon when in the circadian cycle this agent was administered [122]. The mortality was lowest when TNF-α was administered in the second half of the daily activity (in the early and mid-hours during the dark period) and the lowest survival rate was observed in the late hours of the light period (just before awaking). The first human study that investigated the day-night different in the acute phase response was by Pollmächer et al. [37]. They induced 12 young volunteers with LPS endotoxin at 9:00 h and 19:00 h. Significant diurnal variations occurred in the hormonal (ACTH, cortisol) and pyrogenic response to endotoxin, but no differences was seen in the blood plasma levels of TNF-α and IL-6. Marpegan et al. confirmed the result by Halberg et al., and went beyond this to investigate the clinical implications of this day-night difference in the endotoxaemic response [36]. In this study mice were challenged with shock doses of LPS endotoxin at evening (18:00) and at night (04:00); and were monitored for 90 h post-LPS injection. They found that the survival rate was significantly higher at night compared to day.

Several mechanisms can explain the circadian differences in the endotoxaemic response [36]. One mechanism explaining the variable vulnerability to the cytokines levels, is the interaction between clock genes and the LPS endotoxin. In macrophages, more than 8% of the gene transcription of many important pathogen recognition molecules and cytokines oscillates in a rhythmic manner [123]. The gene PER2 is a key molecular component in controlling the circadian rhythm. Takahashi et al. examined the effect of LPS on the expression of the clock genes PER1 and PER2, and found that only PER1 in the periventricular nucleus but not in the SCN was increased at ZT7 compared to ZT17 under forced swimming and at ZT11 compared to ZT21 after LPS injection [124]. Liu et al. showed that PER2-deficient mice had higher survival rate compared with wild type mice in an endotoxaemia induced septic shock model [125]. On the other hand, LPS endotoxin reduces PER2 gene-expression, and in a recent study this gene-suppression was shown to be time-dependent [126]. In humans, the only study examining the effect of LPS on clock genes was made by Haimovich et al. [127]. They found that LPS suppressed as much as 90% of the gene expression of PER1, PER2 and other clock genes in human peripheral blood leukocytes (PBL) for at least 17 hours. Furthermore, they demonstrated that melatonin secretion was not impaired by LPS, indicating that the circadian rhythm between the clock genes in the hypothalamus and

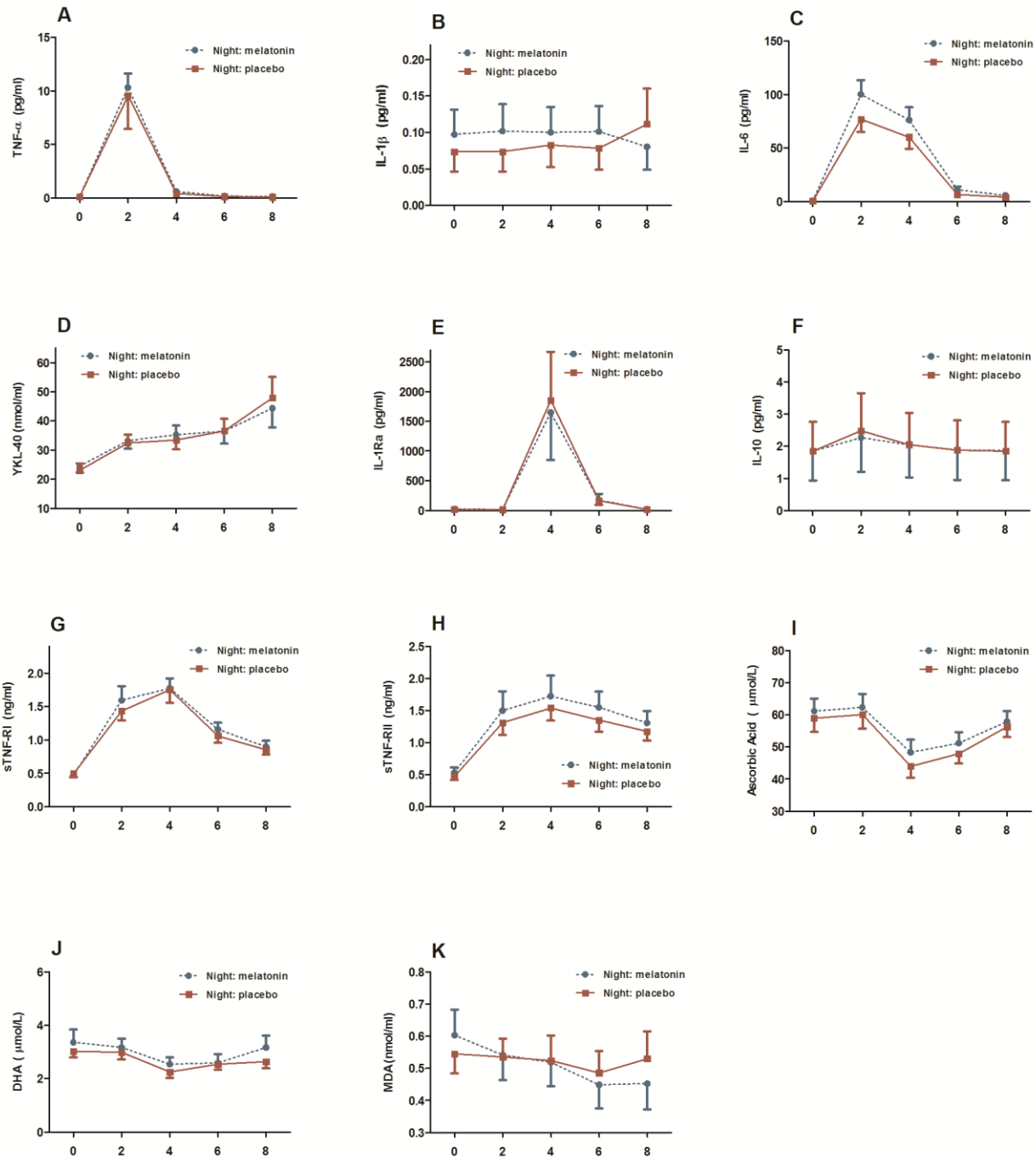


**Figure 7**

Effect of melatonin in nighttime endotoxaemia. Plasma levels of inflammation and oxidation markers. The time point 0 indicates the administration of LPS endotoxin. The endotoxaemia was induced at 24:00. and melatonin (blue curve) or placebo (red curve) was given before the onset of the endotoxaemia. Results from the two-way ANOVA: (1) interaction term (time*intervention) was not significant for any of the markers, (2) between groups was not significant for any of the markers.

clocks genes in PBL were disrupted by endotoxin [127]. Recently, it was shown that several circadian clock genes were suppressed in PBL of surgical intensive care unit patients [128].

Another hypothesis behind the diurnal variation in the observed endotoxaemia is the involvement of the endogenous plasma levels of physiological melatonin and the potent anti-inflammatory hormone cortisol. While melatonin level in plasma peaks in the dark period and is inhibited to almost an undetectable level in the blood during the light period, cortisol levels in plasma have a maximum in the early light hours and is suppressed during the dark period [37]. Thus, because both melatonin and cortisol are potent anti-inflammatory agents, this opposite rhythmicity is very favorable for the anti-inflammatory capacity in the organism. LPS endotoxin has been shown, in animals but not in human, that it can suppress directly the synthesis and secretion of melatonin from the pineal gland [129]. Therefore, the administration of LPS endotoxin during the night will result in a reduced anti-inflammatory capacity compared with daytime endotoxaemia, where the cortisol concentration is highest. The LPS endotoxin tolerance is present 2 weeks after an endotoxaemic response in healthy volunteers, and therefore the lack of day-night difference in the study of Pollmächer et al. [37] might be due to the development of LPS endotoxin tolerance originated from the first exposure to the LPS endotoxin, since the wash-out period was less than 10 days.

The fact that melatonin has been shown to have potent anti-inflammatory and anti-oxidative effects should be considered in the design of studies investigating day-night differences in endotoxaemia. In our study, the endotoxaemia were induced at 12:00 h, where physiological melatonin is undetectable, and at 24:00 h, where the endogenous levels of melatonin are rising to its maximum. Pollmächer et al. induced the endotoxemia at 9:00 h and 19:00 h, thus the onset at night was before the normal increase in the circulating level of melatonin [37]. This might also be the reason for the lack of day-night difference in the inflammatory response seen in this study. Recently, the expression of TLR4-receptors that bind the endotoxin and initiates a downstream signaling was shown not to exhibit circadian rhythmicity [130]. The day-night variation was rather seen in the costimulators (CD80 and CD86) of the receptors in LPS-stimulated human CD14+ monocytes. This indicates that not the receptor exposure but rather the TLR function displays a diurnal rhythmicity [130]. In addition to the previously mentioned theories explaining the observed day-night difference in endotoxaemia, a circadian rhythm in the recognition and signaling systems in the host immune cells might also contribute to this difference.

***Oxidation***

In this PhD thesis, we could demonstrate a day-night difference in the levels of the end-product, MDA, of the lipid peroxidation due to endotoxaemia in human models but not in animal models. The antioxidant ascorbic acid did not show a circadian variation but the superoxide dismutase in the liver tissue had significantly higher activity during daytime compared with night time, thus indicating a higher antioxidant capacity in the liver during the day. To our knowledge there are no previous studies dealing with the day-night difference in the lipid peroxidation and the antioxidant during an immune challenge such as the LPS endotoxaemia. Under normal conditions the levels of MDA has been shown to have a circadian rhythm while other studies question this circadian rhythmicity [131-133]. While the inflammatory response was more pronounced at night, the lipid peroxidation resulted in a higher concentration of MDA in plasma at daytime. This might be due to the day-night difference in the antioxidant capacity of the body, where studies have shown that the SOD and glutathione-transferase have maximum activity during the light phase while glutathione-reductase peaks in the dark phase of the day [134]. This circadian variation of the activity of the antioxidative enzymes depends on the tissue type and the animal species [134].

**The modulatory effect of melatonin on endotoxaemia**

The modulatory effect of melatonin on endotoxaemia has been investigated intensively in animal models, but we were the first to examine this effect in an experimental human model. We also studied the day-night difference in the effect of melatonin both in human and animal models.

***Inflammation***

The human model demonstrated that melatonin at daytime had a beneficial effect by reducing the levels of YKL-40 and IL-1β. In an animal model we could not demonstrate a beneficial effect of melatonin on the circadian levels of IL-6 and IL-10 but we demonstrated that there was a substantial day-night difference in the effect of melatonin on inflammation.

Several studies have demonstrated that melatonin has a powerful anti-inflammatory effect on endotoxaemia. This effect has been demonstrated on the blood plasma levels of the cytokines, decreasing TNF-α, IL-1β, INF-γ, IL-6, IL-8 and IL-12; and increasing IL-10 and IL-1Ra. Melatonin also reduces superoxide production in the aorta and iNOS in the liver [98-101]. It also significantly decreases lung lipid peroxidation and counteracted the LPS induced increase in NO levels in lungs and liver [98,102-104]. Furthermore, melatonin reduced the production of free radicals in the mitochondria by inhibiting complexes 1 and 4 of the electron transport chain [104]. The development of apoptosis due to severe endotoxaemia was also significantly reduced by the administration of melatonin [99]. The effect of melatonin on LPS induced multi organ failure has also been evaluated. In animal models, melatonin counteracted the development of kidney and metabolic dysfunction induced by LPS. Melatonin reversed LPS induced intestinal motility disturbances and normalized the increased lipid peroxidation, iNOS expression and nitrite production in intestinal tissue. In lungs, melatonin prevented the decrease in the $PaO_2$, pulmonary oedema, elevated lung myeloperoxidase activity and lipid peroxidation after LPS.

In humans, melatonin has never been tested on experimental endotoxaemia but rather in clinical settings [48-52]. Through several studies, Gitto & Fulia demonstrated a beneficial effect of melatonin on new-borns with sepsis, infants undergoing surgery, preterm new-borns with bronchopulmonary dysplasia, new-borns with respiratory distress syndrome, and asphyxiated new-borns [48-52]. Melatonin decreased the levels of IL-6, IL-8, TNF-α, white blood cells count, the absolute neutrophil count, and the C-reactive protein, and it increased the levels of platelets to normal values [48-52]. Kücükakin et al. investigated whether melatonin had an effect on the inflammatory response developed under clinical surgical conditions. However, they found no effect of melatonin when given to patients undergoing minor surgery (cholecystectomy) and major surgery (aortic aneurism repair), with respect to IL-6 and C-reactive protein [121,135].

***Oxidation***

We could demonstrate an effect of melatonin on lipid peroxidation, where the concentration of MDA was reduced in animal models but not in human models. Also the antioxidant amount of ascorbic acid was significantly increased by melatonin compared

to placebo in animal models but not in humans. Although melatonin has been investigated intensively in animal models showing a clearly strong antioxidative effect, these results have not yet been confirmed in human studies. Gitto & Fulia could demonstrate an effect on the oxidative damage and oxidative stress in patients undergoing surgery and physiological stress, sepsis, bronchopulmonary dysplasia, respiratory distress syndrome and asphyxia, with respect to MDA, nitrate and nitrite [48-52]. On the other hand, Kücükakin et al. could not demonstrate an effect of melatonin on MDA and ascorbic acid, probably because the doses used were lower [121,135]. Patients undergoing cholecystectomy received 10 mg melatonin infusion intraoperatively [135]. Patients undergoing elective abdominal aorta aneurysm repair received 50 mg melatonin infusion intraoperatively and 10 mg orally for the first three postoperative days [121]. No effect was seen on both studies.

In our study we administered melatonin intravenously in humans, thereby bypassing the liver metabolism and reducing the concentration of the metabolites dramatically. The metabolites of melatonin (AFMK, AMK and 6-hydroxymelatonin), especially AFMK, have been shown to have powerful antioxidant effects [40-42]. The 6-hydroxymelatonin has been shown to have scavenging effects on free radicals but is much less lipophilic than melatonin and therefore cannot cross lipid barriers as easily as melatonin [40]. Furthermore, AFMK and AMK selectively inhibit gene expression of cyclo-oxygenase 2 (COX-2) in vitro [136]. COX-2 is a proinflammatory enzyme that is stimulated by LPS through the signaling from the TLR-4. COX-2 plays a key role in the proinflammatory process since it catalyzes the biosynthesis of prostaglandins (PG) from arachidonic acid. PG plays an important role in inflammation, immune functions, blood vessel dilatation and neurotransmission, resulting in fever, pain and edema during systemic inflammatory responses [87,136,137]. The fact that the metabolites of melatonin can neutralize the free radicals efficiently and inhibit inflammation through different pathways may lead to the question whether effects of metabolites interact with the effect of melatonin, resulting in a synergistic effect.

We did not demonstrate any day-night differences in the effect of melatonin in humans, but in our animal study, the concentrations of IL-6 and IL-10 were altered at daytime but not at nighttime. Furthermore, the amount of ascorbic acid was significantly lower at nighttime compared to daytime. This difference in the effect of melatonin depending on the time of day when melatonin was administered has previously been demonstrated in a study where melatonin was tested on tissue regeneration [138]. Here they found that melatonin given in the morning hours increased collagen capacity in granulation tissue compared with evening hours administration. In a mice study the antitumor effect of melatonin was tested at different times during the day. Melatonin given at night (01:00) reduced the weight of the tumor significantly more compared with melatonin given at midday (13:00) [139]. Furthermore, the amount of melatonin bound to the receptors on tumor cell surface and the clearance rate of melatonin was significantly higher at night compared to midday. This indicates that the pharmacodynamics and pharmacokinetics of melatonin may exhibit a day-night variation [139].

**FUTURE PERSPECTIVES**

The relationship between circadian rhythm and the pathophysiology of the diseases are becoming more and more evident, and intensive research exploring this association and examination of the molecular mechanisms behind this association are taken place in these years. The main remaining questions are whether this association and day-night difference in the pathophysiological processes are clinically relevant, i.e. if the differences in the levels of cytokines between day and night shown in this thesis influence the morbidity and the mortality in patients. . Thus, it is also unknown whether we need to adapt pharmacological interventions to this internal rhythmicity of the body's capacity to response to a septic challenge. There is also need for studies examining the molecular mechanisms producing this rhythmicity. It is unknown if the clock genes play a role in the rhythmicity, or how endogenous anti-inflammatory and antioxidative components in the body interfere with the rhythmic inflammatory response.

Melatonin's effect on reducing the oxidative damage and inflammatory response is an obvious research area for future clinical trials. In animal models, the effect of melatonin has been tested in numerous pathophysiological conditions with oxidative and inflammatory stress, indicating a clearly powerful inhibitor of ischemic-reperfusion injury, radioactive damage, UV-mediated damage, tissue regeneration, neurodegenerative disorders, rheumatic disorders, and metabolic disorders [40]. Therefore, it is time to enlarge this research area to involve human experimental studies and human clinical trials, investigating both the prophylactic and therapeutic effects of melatonin. The pharmacodynamics of melatonin is a major challenge, because the liver metabolism of melatonin can differ as much as 37-fold in normal healthy individuals. Furthermore, the metabolites of melatonin, which also exert potent anti-inflammatory and antioxidative effects, should be considered in future study designs by oral or intravenous administration of melatonin. Also the interaction between melatonin and the metabolites should be examined. Finally, the chronopharmacology of melatonin is very interesting; whether the effect of melatonin depends on time of the day it is administered.

**SUMMARY**

The circadian rhythm in pathophysiological conditions has been known for many years. The symptoms in asthma bronchiale and the incidence of sudden cardiac death, pulmonary thromboembolism, and acute myocardial infarction all exhibit a rhythmic pattern through the day/night. In the immune system, a rhythmic cycle has also been described, and the oscillations exist both under normal, unstimulated conditions, and also when the immune system faces a challenge. The last mentioned is only examined in in vitro and ex vivo studies. Little is known about the circadian rhythm in the immune response in in vivo settings, where few studies have demonstrated that a circadian pattern might exist.

In this thesis the circadian variation in the response to an LPS endotoxin challenge was investigated in rats and in humans. In rats, the response after LPS revealed a significantly higher inflammatory and oxidative response during the dark period compared with the light period of the day. We found that the cytokines levels in the blood plasma differed significantly between a day and night onset of the endotoxaemia. Also the antioxidant enzyme activity of SOD was significantly altered. The same rhythmic pattern was confirmed in a human endotoxaemia model, except that the lipid peroxidation was higher during daytime endotoxaemia.

Melatonin, an endogenous circadian synchronizer secreted from the pineal gland, has potent antioxidative and anti-inflammatory effects. In rats, we demonstrated that melatonin, both in daytime and nighttime endotoxaemia, had a strong inhib-

iting effect on lipid peroxidation by reducing the levels of MDA, and melatonin increased the antioxidants' capacity. The effect on the inflammatory response showed great time dependence. In a human endotoxaemia model, the beneficial effect of melatonin was seen in the daytime endotoxaemia but not in night time endotoxaemia, with respect to the inflammatory response but not the lipid peroxidation and antioxidants.

Future trials should investigate whether the observed diurnal difference in the endotoxaemia effects exists in clinical settings, e.g. septic patients, and whether the difference has clinical implications with respect to morbidity and mortality. It is also of importance to study the molecular mechanisms resulting in this circadian rhythmicity. Finally, the effect of melatonin in clinical settings should be examined, taking into consideration the chronopharmalogical differences seen in the effect of melatonin.

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