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Melatonin in septic shock: Some recent concepts

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Abstract

Melatonin is a versatile molecule, synthesized not only in the pineal gland, but also in many other organs. Melatonin plays an important physiological role in sleep and circadian rhythm regulation, immunoregulation, antioxidant and mitochondrial-protective functions, reproductive control and regulation of mood. Melatonin has also been reported as effective in combating various bacterial and viral infections. Melatonin is an effective anti-inflammatory agent in various animal models of inflammation and sepsis and its anti-inflammatory action has been attributed to inhibition of nitric oxide synthase with consequent reduction of peroxynitrite formation, to the stimulation of various antioxidant enzymes thus contributing to enhance the antioxidant defense and to protective effects on mitochondrial function and in preventing apoptosis. In a number of animal models of septic shock, as well as in patients with septic disease, melatonin reportedly exerts beneficial effects to arrest cellular damage and multiorgan failure. The significance of these actions in septic shock and its potential usefulness in the treatment of multiorgan failure are discussed.

Key words: melatonin; septic shock; antioxidants; cytokines; nitric oxide.

Introduction

Melatonin is a major secretory product of the pineal gland released every day at night. In all mammals, circulating melatonin is synthesized primarily in the pineal gland. In addition, melatonin is also locally found in various cells, tissues and organs including lymphocytes, human and murine bone marrow, the thymus, the gastrointestinal tract, skin and the eyes where it plays either an autocrine or paracrine role (see for ref. [1,2]). Both in animals and in human beings, melatonin participates in diverse physiological functions signaling not only the length of the night (and thus the time of the day or the season of the year) but also enhancing free radical scavenging, the immune response and cytoprotective processes.

In several animal models melatonin has been identified to protect against bacterial, viral and parasitic infections presumably by acting through a variety of mechanisms, like immunomodulation or direct or indirect antioxidant activity (see for ref. [3]). Melatonin is a powerful antioxidant that scavenges superoxide radicals as well as other radical oxygen species (ROS) and radical nitrogen species (RNS) and that gives rise to a cascade of metabolites that share its antioxidant properties. Melatonin also acts indirectly to promote gene expression of antioxidant enzymes and to inhibit gene expression of prooxidant enzymes [2].

Septic shock, the most severe problem of sepsis, is a lethal condition caused by a pathogen-induced long chain of sequential intracellular events occurring in immune cells, epithelium, endothelium, and the neuroendocrine system (see for ref. [4]). The lethal effects of septic shock are associated with the production and release of numerous pro-inflammatory biochemical mediators like cytokines, nitric oxide (NO), ROS and RNS radicals, together with development of massive apoptosis.

Melatonin has been shown to be beneficial for reversing symptoms of septic shock [5]. Melatonin had significant anti-inflammatory properties presumably by decreasing the synthesis of pro-inflammatory cytokines like tumor necrosis factor (TNF)- α and by suppressing inducible NO synthase (iNOS) gene expression. Melatonin also exerts a strong antiapoptotic effect (see for ref. [2]). This review article is focused on the significance of melatonin in septic shock and its potential utility to treat multiorgan failure. Published studies on animal models of inflammation and sepsis are summarized in Supplemental Tables 1 and 2.

In next sections we will review some of those studies with the aim of exemplifying the potential therapeutical use of melatonin in inflammation and septic shock.

Melatonin in lipopolysaccharide (LPS)-induced inflammation

(PLEASE, REFER TO SUPPLEMENTAL TABLE 1 HERE)

The first evidence for melatonin in controlling LPS-induced damage was provided by Sewerynek and coworkers in rats [6]. They reported a reduction in LPS-induced oxidative insult after melatonin administration, as evidenced by decreased hepatic malondialdehyde (MDA) and 4-hydroxyalkenal (4-HDA) [6].

Melatonin prevents LPS-induced endotoxemia presumably by reducing circulating TNF- α levels, superoxide production in the aorta, and iNOS in the liver [7]. Melatonin (10-60 mg/kg) administered i.p. to rats before and/or after LPS significantly decreased lung lipid peroxidation and counteracted the LPS-induced increase of NO levels in lungs and liver in a dose-dependent manner [8]. It also prevented LPS-induced metabolic alterations.

The activation of mitochondrial NOS can be a crucial trigger for initiation of the chain of events leading to septic shock [9]. The mitochondria express constitutive and inducible forms of NOS, the latter causing mitochondrial respiratory inhibition and failure. The protective role of melatonin against the enhancing effects of LPS on mitochondrial iNOS and the activity of respiratory complexes in liver and lung mitochondria was evaluated in young and old rats [10]. Melatonin administration (60 mg/kg, i.p.) effectively counteracted LPS-induced inhibition of complexes I and IV of the electron transport chain and decreased mitochondrial NOS activity and NO production, thereby preventing LPS toxicity [10]. The survival rate of LPS-injected mice improved from 0% in controls to 48% and 86% following melatonin administration (2 mg/kg) 3 and 6 later respectively [11].

The effect of melatonin in preventing septic shock is complex. Apart from acting on the local sites of inflammation melatonin also exerts its beneficial actions through a multifactorial pathway including its effects as immunomodulator, antioxidant, and antiapoptotic agent. This is exemplified by the study performed by Carrillo-Vico et al. in mice [12]. It was reported that i.p. administered melatonin (10 mg/kg) 30 min prior to and one hour after LPS injection markedly

protected the mice from the lethal effects of LPS with 90 % survival rates for melatonin versus 20 % in LPS-injected mice after 72 h. LPS induced the increase of nitrite/nitrate and lipid peroxidation levels in brain and liver. Melatonin administration increased the levels of the anti-inflammatory cytokine interleukin (IL)-10 and decreased the concentration of pro-inflammatory mediators like TNF- α , IL-12, and interferon- γ in the local site of LPS injection [12]. Morphological evaluation of the apoptotic process showed that melatonin decreased the LPS-induced programmed cell death in spleen [12]. Melatonin's antiapoptotic action was attributed to its stimulatory effect on IL-10 levels, since IL-10 antiapoptotic action had already been demonstrated.

The effect of senescence on LPS-induced multiorgan failure and the efficacy of melatonin treatment to modify this condition was evaluated in rats [13]. iNOS expression and activity, nitrite content, lipoperoxidation levels, and serum markers of liver, renal and metabolic dysfunction, were measured. An age-dependent increase in iNOS activity, NO content and lipoperoxidation levels was observed, and these changes were augmented further by LPS [13]. Melatonin decreased the expression and activity of iNOS, reducing NO and lipoperoxidation levels to basal values in both LPS-treated groups. Liver, kidney and metabolic dysfunctions were also significantly higher in aged rats and further increased by LPS. Melatonin treatment counteracted all these alterations in young and aged rats [13]. These findings are significant since the susceptibility of elderly patients to septic shock and multiorgan failure is much greater than in young individuals. The alteration of the sleep/wake cycle may be one of the factors explaining this susceptibility in old individuals. Relevant to this melatonin (5 mg/kg i.p.) attenuated the alveolar damage caused by LPS and counteracted the reduced the levels of Bcl-XL and procaspase-3 seen in sleep-deprived mice [14]. Melatonin also prevented the increase of cell death and reduced the elevated higher levels of MDA in lungs of sleep-deprived mice [14].

It is well known that during sepsis ileus and mucosal cell barrier dysfunction occurs as one of its most frequent complications. Ileus, by promoting intestinal stasis, bacterial overgrowth, and bacterial translocation may cause secondary infections and multiple organ failure. By using the LPS model, the beneficial effects of melatonin in preventing gastrointestinal disturbances were studied in mice [15]. Mice treated with LPS exhibited reduced

gastric emptying of solid beads and altered distribution of glass beads throughout the GI tract. Melatonin (10 mg/kg i.p.) reversed LPS-induced motility disturbances. Melatonin also normalized the altered lipid peroxidation, p38 mitogen-activated protein kinase (MAPK) activation, nuclear factor- κ B activation, iNOS transcription and expression and nitrite production in intestinal tissue from septic mice [15]. Melatonin is thus a molecule with therapeutic potential for the treatment of systemic inflammation since it interferes at the earliest step of activation of the oxidative and pro-inflammatory cascade. Melatonin and its metabolites may function as modulatory agents during the inflammatory process and have the potential to be a new class of anti-inflammatory agents with specificity for cyclooxygenase-2 and iNOS enzymes [16]. Melatonin treatment also reduced myeloperoxidase activity and MDA levels [17]. .

The possible protective effect of melatonin in LPS-induced pulmonary inflammation and lung injury was evaluated in rats [18]. Melatonin (10 mg/kg, i.p.) given 30 min prior to LPS prevented the decrease in PaO₂ and the lung injury caused by the endotoxin. Melatonin decreased pulmonary edema, the elevated lung MPO activity and lipid peroxidation after LPS. The increase of the pro-inflammatory cytokine TNF- α levels in pulmonary tissue given by LPS was also prevented by melatonin while the levels of the anti-inflammatory cytokine IL-10 augmented. The decrease in LPS-induced the pulmonary edema, lipid peroxidation and the infiltration of neutrophils in lung tissue was interpreted in terms of the NF- α inhibition and IL-10 stimulation brought about by melatonin [18].

Severe infection in diabetic patients often leads to multiorgan failure. In a study conducted to assess the protective effects of melatonin in LPS-injected rats turned diabetic by streptozotocin administration, LPS significantly increased the serum levels of TNF- α and IL-6 in normal and diabetic rats and augmented plasma corticotropin-releasing hormone, ACTH and corticosterone [19]. Both 0.1 and 1 mg/kg melatonin doses significantly decreased serum levels of TNF- α and IL-6 in LPS-treated rats. Significant inhibitory effects of melatonin (1 mg/kg) were also observed on the hypothalamic-pituitary- adrenal axis [19]. Previous studies in diabetic rats indicated that melatonin was effective to restore normal vascular responses [20]. Therefore, melatonin treatment may help to prevent the vicious cycle of hyperglycemia and stress factors such as severe infection in diabetic patients.

Melatonin in non-LPS animals models of septic shock

(PLEASE, REFER TO SUPPLEMENTAL TABLE 2 HERE)

Short-term melatonin administration (10 mg/kg i.p.) after hemorrhage significantly improved survival in animals subjected to a subsequent septic challenge by the cecal ligation and puncture (CLP) procedure [21]. In these mice melatonin administration increased the survival rate by 28% as compared to vehicle-treated animals.

In another experimental model for septic shock, i.e. the systemic administration of zymosan A that causes a massive the release of pro-inflammatory mediators like TNF- α , IL-6, prostaglandins (PGs) , NO and ROS, a 100% mortality was observed whereas the simultaneous administration of zymosan and melatonin (0.8 mg/kg) resulted in only 27% mortality rate [22].

The CLP model of sepsis was used to further understand the possible involvement of mitochondrial NOS and melatonin in the pathophysiology of sepsis by examining the changes in mitochondrial constitutive and iNOS activity and mitochondrial function in skeletal muscles of wild-type (iNOS^{+/+}) and iNOS knockout mice (iNOS^{-/-}) [23]. Four doses of melatonin (30 mg/kg) were injected i.p. In iNOS^{+/+} mice, but not in iNOS^{-/-} mice, sepsis increased mitochondrial NOS activity. Melatonin administration counteracted sepsis-induced mitochondrial iNOS activity in iNOS^{+/+} mice, but did not affect mitochondrial constitutive NOS activity in either type of mice [23]. Mitochondrial nitrite significantly increased in iNOS^{+/+} mice after sepsis while melatonin treatment reduced nitrite levels to control values. Lipid peroxidation, which was increased in septic iNOS^{+/+} mice, decreased significantly after melatonin administration. Sepsis significantly reduced the mitochondrial content of total glutathione (GSH) in iNOS^{+/+} mice and increased the oxidized glutathione (GSSG)/GSH ratio indicating a loss of reduced GSH. These changes in mitochondrial GSH pool during sepsis were counteracted by melatonin administration [23]. As far as the electron transport chain, complex I ,II, III and IV activities were significantly reduced in septic NOS^{+/+} mice by about half and the administration of melatonin not only prevented the inhibition of complex activities induced by sepsis but also increased their activities above their basal values [23].

Although extensively studied, the pathophysiology of sepsis-associated multiorgan failure remains undefined [4,24]. It has been proposed that a key defect in sepsis is the disruption of

oxidative phosphorylation within mitochondria [25]. The result is an inability of the cell to use molecular oxygen for ATP production, despite adequate oxygen availability. Melatonin normalized the production of ATP in iNOS^{+/+} septic mice, without affecting iNOS^{-/-} animals [26].

The efficacy of melatonin to prevent intraperitoneal sepsis and the associated multiple organ dysfunction syndrome was evaluated in rats subjected to the CLP procedure [27]. Melatonin was administered 3, 6, and 12 h after CLP. The pressor response to norepinephrine (NE) was assessed at 0, 3, 9 and 18 h after CLP surgery. Animals that received CLP alone showed a significantly progressive decrease in mean arterial blood pressure from 9 to 18 h (i.e. from about 120 to 70 mmHg). In the animals that received CLP plus melatonin (0.3 mg/kg i.v. at 3 and 9 h after CLP) the delayed fall in blood pressure was prevented [27]. The administration of melatonin completely restored the NE-induced vasomotor response back to normal. With regard to biochemical indexes of liver dysfunction, the rise in plasma glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase caused by CLP was prevented by melatonin treatment, as it was the increase in creatinine, blood urea nitrogen and lactate dehydrogenase (indicators of renal failure and cellular damage) in response to CLP [27].

Several cytokines released during sepsis, especially IL-1 β , directly acts on blood vessels inducing vasodilatation through rapid production of platelet-activating factor and NO. In CLP rats melatonin treatment diminished plasma NO and IL-1 β concentrations, aortic superoxide levels and the infiltration of polymorphonuclear neutrophils in lung and liver [27]. Therefore, Wu et al. attributed the beneficial effects of melatonin in the CLP septic model to inhibition of IL- β and NO production, O₂⁻ formation and polymorphonuclear infiltration in organs [27]. In a previous study it had been observed that melatonin (10 mg/kg, i.p.) 30 min prior to and 6 h after CLP counteracted the inhibition of in vitro ileal and bladder contractility caused by sepsis [28]. Considering that micromolar melatonin concentrations could be locally achieved through production by activated immune competent cells, extra-pineal melatonin could have a protective effect against tissue injury in multiple organ dysfunction syndrome [29].

Melatonin studies in septic patients

Several studies have measured melatonin levels in critically ill patients to find out a possible correlation between melatonin and intensity of septic shock. In one of those studies

carried out in intensive care unit (ICU) patients, 17 septic ICU patients, 7 ICU nonseptic patients and 21 controls were examined [30]. 6-Sulfatoxymelatonin was determined in urine samples taken at 4-h intervals over a total period of 24 h. Urinary 6-sulfatoxymelatonin exhibited significant circadian periodicity in only 1/17 septic patients versus 6/7 in non septic patients and in 18/23 in normal controls. The phase amplitude (an index of the maximal levels attained at peak concentrations) was significantly lower in septic patients. In sepsis survivors, 6-sulfatoxymelatonin excretion profiles tended to normalize, but still lacked a significant circadian rhythm at ICU discharge [30].

In another study melatonin levels in blood and urine were studied over 3 consecutive days in 8 critically ill patients during deep sedation and mechanical ventilation at the ICU [31]. The circadian rhythm of melatonin release was abolished in all but one patient, who recovered much more quickly than the others [31].

Biochemical markers for the circadian rhythm were studied in 16 patients treated at the ICU of two regional hospitals in Sweden [32]. All urine excreted between 07:00 and 22:00 hours (day) and between 22:00 and 07:00 hours (night) was collected and sampled throughout the entire ICU period (median, 10 days) for the excretion 6-sulfatoxymelatonin and free cortisol. Overall excretion of 6-sulfatoxymelatonin was lower and cortisol excretion higher than reported for healthy reference populations [32]. Mechanical ventilation was associated with a markedly lower 6-sulfatoxymelatonin excretion (median, 198 ng/h) compared with periods without such help (555 ng/h), whereas infusion of adrenergic drugs increased 6-sulfatoxymelatonin excretion significantly. Five patients (31%) showed a virtually absent melatonin excretion for 24 h or more. The diurnal rhythms were consistently or periodically disturbed in 65% and 75% of the patients [32].

Perras et al. measured serum melatonin concentrations at 02:00 h in the first night in hospital in 302 patients consecutively admitted to the ICU [33]. Correlations between illness severity (Acute Physiology And Chronic Health Evaluation, APACHE, II score; Therapeutic Intervention Scoring System, TISS) and melatonin levels were assessed. Overall analysis for the whole group of patients revealed no or very weak correlation between nocturnal serum melatonin levels and illness severity. In contrast, analysis of subgroups indicated that in the 14 patients with severe sepsis APACHE and TISS scores were correlated negatively with nocturnal

melatonin concentrations. In contrast, melatonin levels and illness severity were not correlated in patients admitted for coronary syndrome, intoxication, gastrointestinal bleeding, pneumonia or stroke [33].

The alteration of the sleep-wake cycle, the augmented oxidative/nitrosative stress and the altered inflammatory reaction seen in patients with septic shock render them suitable for melatonin therapy [34]. To assess in critically ill patients receiving mechanical ventilation the effect of exogenous melatonin on nocturnal sleep quantity, a randomized double-blind placebo-controlled trial including 24 patients who had undergone a tracheostomy was performed [35]. Oral melatonin (10 mg) or placebo was administered at 21:00 h for 4 nights. Nocturnal sleep was evaluated using the bispectral index (a signal-processing electroencephalographic technique). Actigraphy and subjective assessment of sleep were also employed. Nocturnal sleep time was 2.5 h in the placebo group and melatonin use was associated with a 1-h increase in nocturnal sleep [35]. Based on the supraphysiological melatonin levels detected in plasma at the end of the night the authors concluded that a lower amount of melatonin (1-2 mg) would probably be enough to improve sleep. However, amounts of melatonin in the 10-mg range or higher should be needed to warrant the effect on reduction of ischemic reperfusion injury, prevention of multi-organ failure or treatment of sepsis.

From a different perspective, namely to curtail oxidative stress, a number of clinical studies performed by Gitto and co-workers have showed that melatonin reduces oxidative stress in newborns with sepsis, distress or other conditions where there is excessive ROS production (see for ref. [36]). In the first of these studies, a product of lipid peroxidation, MDA and the nitrite/nitrate levels were measured in the serum of 20 asphyxiated newborns before and after treatment with melatonin given within the first 6 h of life. Ten asphyxiated newborns received a total of 80 mg of melatonin (8 doses of 10 mg each separated by 2-h intervals) orally. One blood sample was collected before melatonin administration and two additional blood samples (at 12 and 24 h) were collected after giving melatonin. Serum MDA and nitrite/nitrate concentrations in newborns with asphyxia before treatment were significantly higher than those in infants without asphyxia. In the asphyxiated newborns given melatonin, there were significant reductions in MDA and nitrite/nitrate levels. Three of the 10 asphyxiated children not given

melatonin died within 72 h after birth; none of the 10 asphyxiated newborns given melatonin died [36].

In a second study a total of 20 mg melatonin was administered orally in two doses of 10 mg each, with a 1-h interval. The changes in the clinical status and the serum levels of the lipid peroxidation products MDA and 4-HDA were recorded in 10 septic newborns treated with the antioxidant melatonin given within the first 12 h after diagnosis. Ten other septic newborns in a comparable state were used as "septic" controls, while 10 healthy newborns served as normal controls. Serum MDA + 4-HDA concentrations in newborns with sepsis were significantly higher than those in healthy infants without sepsis and they were significantly reduced by melatonin. Melatonin also improved the clinical outcome of the septic newborns as judged by measurement of sepsis-related serum parameters after 24 and 48 h [36].

Gitto et al. also examined whether melatonin treatment would lower IL-6, IL-8, TNF- α and nitrite/nitrate levels in 24 newborns with respiratory distress syndrome III or IV grade diagnosed within the first 6 h of life. Compared with the melatonin-treated respiratory distress syndrome newborns, in the untreated infants the concentrations of IL-6, IL-8, and TNF- α were significantly higher at 24 hours, 72 hours, and at 7 days after onset of the study. In addition, nitrite/nitrate levels at all time points were higher in the untreated respiratory distress syndrome newborns than in the melatonin-treated babies. Following melatonin administration, nitrite/nitrate levels decreased significantly, whereas they remained high and increased further in the respiratory distress syndrome infants not given melatonin.

Pro-inflammatory cytokines (IL6, IL-8 and TNF- α) and the clinical status were examined in 110 preterm newborns with respiratory distress syndrome ventilated before and after treatment with melatonin. When comparing serum levels of IL-6, IL-8 and TNF- α for two groups, melatonin treatment clearly had anti-inflammatory effects [36]. In conclusion, these studies indicate that melatonin lowers IL-IL-6, IL-8, TNF- α and nitrite/nitrate levels and modifies serum inflammatory parameters in surgical neonates improving their clinical course.

Conclusion

Active research continues to define the principal alterations in sepsis, though significant challenges remain before this devastating process is understood and conquered. Melatonin has

entered this arena because it has a promise as an appropriate add-on pharmacological tool in sepsis. Although understanding of the melatonin 's action in the pathogenesis of septic shock is yet to be achieved, studies so far points out that melatonin through its immunomodulatory, antioxidant and antiapoptotic actions may exert beneficial effects in septic shock and multiorgan failure. However, larger randomized controlled clinical trials are necessary to confirm the potential benefits of melatonin therapy before it can be routinely used in the postoperative or critically ill patients [37].

Disclosure

S.R. Pandi-Perumal is a stockholder and the President and Chief Executive Office of Somnogen Inc., a New York Corporation. He declared no competing interests that might be perceived to influence the content of this article. All remaining authors declare that they have no proprietary, financial, professional, nor any other personal interest of any nature or kind in any product or services and/or company that could be construed or considered a potential conflict of interest that might have influenced the views expressed in this manuscript.

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Supplemental Table 1

Protective effects of melatonin on LPS-induced inflammation

Animal model	Parameters studied	Effects observed	References
LPS in rats.	Hepatic lipid peroxidation. Hepatic GSH and oxidized GSSG levels. Histological examination of liver.	Melatonin (1 mg/kg i.p.) abolished LPS-induced increase in lipid peroxidation, exaggerated the rise in total GSH and reversed the increase in GSSG concentration and the histological degenerative changes seen in liver after LPS.	[1]
LPS in phenobarbital-treated rats.	GSH and GSSG concentration, the activity of the antioxidant enzyme GSH-Px in brain and liver, and the content of cytochrome P450 reductase in liver were measured.	The injection of melatonin (4 mg/kg i.p.) every hour for 4 h after LPS decreased the high levels of GSSG and enhanced the depressed activity of GSH-Px brought about by LPS but did not affect cytochrome P450 reductase.	[2].
LPS in mice.	Mortality rate. Inflammatory cytokine levels. Macrophage NO generation. Plasma nitrate concentration.	Melatonin (2 mg/kg i.p.) protected mice treated with a lethal dose of LPS especially when given 3 to 6 hr after LPS. This effect did not involve inhibition of inflammatory cytokines or macrophage NO generation.	[3]
LPS in rats.	Genotoxicity was tested using micronuclei as an index in bone marrow and peripheral blood cells.	Melatonin (5 mg/kg i.p.) injected prior to LPS administration and thereafter at 6 h intervals prevented the increase in number of micronucleated polychromatic erythrocytes after LPS.	[4]
LPS in rats.	Serum levels of AST, ALT, gamma-glutamyl-transferase, alkaline phosphatase, creatinine, urea, uric acid, glucose, cholesterol and triglycerides. Lung lipid peroxidation and LPS-induced NO levels in lungs and liver. Expression of iNOS mRNA in lungs and liver.	Melatonin (10-60 mg/kg) was i.p. injected before and/or after LPS. Endotoxemia was associated with liver and renal dysfunction as shown by the abnormality of various parameters tested. Melatonin (20 and 60 mg/kg) counteracted all the effects of LPS. Melatonin decreased lung lipid peroxidation and counteracted the LPS-induced NO generation in lungs and liver. Expression of iNOS mRNA in lungs and liver decreased after melatonin.	[5]
LPS in mice.	Mesenteric blood flow. Serum TNF- α concentration.	At 1 to 100 mg/kg melatonin decreases LPS-induced TNF- α production but has no protective effect on LPS-induced decrease of mesenteric blood flow. Rather, melatonin directly reduces it.	[6].
LPS in rats.	Mortality rate, blood pressure responses to NE in vivo and the infiltration of polymorphonuclear neutrophils into the liver were assessed. Serum TNF- α and nitrite levels, expression of iNOS in liver and superoxide production by aorta were also measured.	Melatonin (1 – 3 mg/kg i.v.) prevented circulatory failure in rats with LPS endotoxemia and improves survival in mice treated with a lethal dose of LPS. Melatonin inhibited (i) the release of TNF- α in plasma, (ii) the expression of iNOS in liver, and (iii) aortic superoxide production.	[7]

Animal model	Parameters studied	Effects observed	References
LPS in rats.	Mitochondrial NOS and respiratory complexes activity in liver and lung mitochondria were measured in young and old rats.	Melatonin administration (60 mg/kg, i.p.) prevented the LPS toxicity and decreased mitochondrial NOS activity and NO production. Melatonin also counteracted LPS-induced inhibition of complexes I and IV. In general, the actions of melatonin were stronger in older animals	[8].
LPS in mice and rats.	Mortality rate.	A single dose of 5 mg/kg of melatonin or N-acetylserotonin simultaneously injected with LPS markedly protected mice from the lethal effect of LPS with survival rates of 90% and 95% for melatonin and N-acetylserotonin, respectively.	[9]
LPS in rats.	Red cell mechanical behavior was tested using a laser diffraction shearing device. Erythrocyte GSH-Px activity was also measured.	Melatonin (10 mg/kg i.p.) counteracted the impaired erythrocyte deformability caused by LPS. GSH-Px activity was significantly enhanced in LPS rats and melatonin administration further stimulated it in erythrocytes.	[10].
LPS in rats.	Phenylephrine vascular response in vivo and in aorta rings collected from rats treated with LPS. Serum nitrite/nitrate levels. Immunohistochemical determination of iNOS, nitrotyrosine, and poly (ADP-ribose) synthetase (PARS).	Melatonin (30 mg/kg i.p.) pretreatment prevents the hyporeactivity to phenylephrine in vivo and in aorta rings collected from rats treated with LPS. Melatonin inhibited iNOS protein expression and reduced NO production and nitrotyrosine formation in aorta. Melatonin inhibited nuclear PARS activation in vascular tissue.	[11].
LPS plus BCG in mice.	In Kupffer cells and hepatocytes levels of ALT, AST and NO, content of MDA and activity of SOD, were measured. TNF- α and IL-1 levels were also measured. Hepatic tissue sections were stained with for light microscopic analysis.	Immunological liver injury induced by BCG+LPS was significantly decreased by melatonin (0.25, 1.0, 4.0 mg/kg). MDA content was decreased and SOD in liver homogenates was upregulated by melatonin. Pro-inflammatory mediators (TNF- α , IL-1, NO) in serum and liver homogenates were significantly reduced by melatonin. Histological examination demonstrated that melatonin could attenuate the area and extent of necrosis, and reduce the immigration of inflammatory cells.	[12]
LPS in rats.	Red cell mechanical behavior was tested using a laser diffraction shearing device. Erythrocyte NO levels and lipid peroxidation were also measured.	Melatonin (10 mg/kg i.p.) counteracted the impaired erythrocyte deformability and increased NO and lipid peroxidation caused by LPS.	[13]

Animal model	Parameters studied	Effects observed	References
LPS in mice.	Tissue and serum levels of TNF- α , IL10, IL-12 and IFN- γ . Nitrite/nitrate and lipid peroxidation levels in brain and liver. Apoptosis in splenocytes.	I.p. administered-melatonin (10 mg/kg) 30 min prior, and 1 hr after i.p. LPS injection (0.75 mg/animal) protected mice from the LPS lethal effects (90% survival rates for melatonin, 20% for LPS-injected mice after 72 hr). Melatonin reduced the increase in LPS-induced pro-inflammatory cytokine levels (TNF- α , IL-12 and IFN- γ) while augmented the anti-inflammatory cytokine IL-10. Melatonin inhibited LPS-induced nitrite/nitrate and lipid peroxidation levels in brain and liver and counteracted the sepsis-associated apoptotic process in spleen.	[14]
LPS in rats.	Contraction response to phenylephrine and the endothelium-dependent relaxation response to acetylcholine were measured in thoracic aorta and pulmonary artery by the isolated artery ring technique. SOD activity and MDA production in arterial tissues were also measured.	Melatonin (5 mg/kg, i.p.) given 30 min before and 60 min after LPS partially reversed the effect of LPS on arterial contractility and arterial SOD and MDA.	[15]
LPS in pregnant mice.	LPS-induced intra-uterine fetal death and growth retardation by measuring number of live fetuses, dead fetuses and resorption sites. Crown-rump and tail lengths were examined to evaluate skeletal development. LPS-induced lipid peroxidation in maternal liver was also assessed.	Pregnant mice received an i.p. LPS on gestational day 15-17. Intra-uterine fetal death and intra-uterine growth retardation was partially reversed by 5 or 10 mg/kg of melatonin simultaneously and 3 hr after LPS. The addition of 10 mg/kg of melatonin 18 hr before LPS almost blocked LPS-induced effects. Pre- plus post-treatments with melatonin significantly attenuated LPS-induced lipid peroxidation in maternal liver.	[16].
LPS in old rats.	iNOS expression and activity, nitrite content and lipoperoxidation levels were measured in liver and kidney. Serum levels of AST, ALT, alkaline phosphatase, total bilirubin and γ -glutamyltransferase were measured to assess hepatic damage. Renal dysfunction and failure were assessed by measuring the serum levels of creatinine, urea and uric acid. Metabolism impairment was assessed by measuring the changes in serum levels of cholesterol and triglycerides, glucose and albumin.	Melatonin (60 mg/kg i.p.) prevented the increase in iNOS activity, NO content and lipoperoxidation levels and the indicators of multiorgan failure after LPS seen in young and old rats.	[17]
LPS in infant rats.	Measurement of SOD and GSH-Px activities, and TBARS levels. TUNEL procedure to assess intestinal apoptosis.	Melatonin (10mg/kg i.p.) given to 7-day-old rat pups injected with LPS prevented the increase in the TBARS levels, and increased the activities of antioxidant enzymes and attenuated apoptotic cell death in both intestinal epithelium and lamina propria.	[18]

Animal model	Parameters studied	Effects observed	References
LPS in D-galactosamine-sensitized mice.	Serum ALT and hepatic GSH-Px and glutathione reductase activities were measured. Hepatic apoptosis was assessed by inhibition of caspase-3 activities and attenuation of DNA laddering. Serum TNF- α , hepatic GSH levels and hepatic NO production were also measured. Histological sections of liver were examined.	When given together with a sublethal dose of LPS, D-galactosamine sensitizes animals to produce apoptotic liver injury with severe hepatic congestion, resulting in rapid death. Melatonin (5 mg/kg, i.p.) 30 min before LPS + D-galactosamine followed by another two doses (2.5 mg/kg, i.p.) prevented all the biochemical and histological changes of apoptotic liver injury and hepatic congestion, except the augmented NO production.	[19]
LPS in pregnant mice.	TNF- α , IL-1 β , IL-6 and IL-10 levels were measured in maternal serum, amniotic fluid, fetal liver and fetal brain.	Pregnant mice were injected with melatonin (5.0 mg/kg, i.p.) 30 min before LPS on gestational day 17. Melatonin pretreatment significantly attenuated LPS-evoked elevation of TNF- α in maternal serum and fetal brain. On the contrary, melatonin augmented LPS-induced increase in IL-10 in maternal serum and fetal liver. Melatonin had no effect on LPS-evoked IL-1 β and IL-6 in maternal serum and amniotic fluid.	[20]
LPS in mice.	Measurement of gastric emptying and geometrical center with solid beads. Determination of lipid peroxidation, MAPK activation, nuclear factor- κ B activation, iNOS transcription and expression and nitrite production in intestinal tissue.	Melatonin (10 mg/kg i.p.) reversed LPS-induced motility disturbances. Melatonin decreased lipid peroxidation, MAPK activation, nuclear factor- κ B activation, iNOS transcription and expression and nitrite production in intestinal tissue from septic mice.	[21]
LPS in sleep-deprived mice.	Histological examination of lungs. Measurement of lung MDA levels. Western blot analysis of Bcl-XL and procaspase-3. TUNEL staining in lungs.	Melatonin (5 mg/kg i.p.) attenuated the alveolar damage caused by LPS and counteracted the reduced the levels of Bcl-XL and procaspase-3. Melatonin also prevented the increase of cell death and reduced the elevated MDA level in lungs of sleep-deprived mice.	[22]
LPS in rats.	Optical microscopy was performed to examine pathological changes in lungs and lung injury score was assessed. Blood gas analysis was carried out. MPO activity, MDA concentrations and TNF- α and IL-10 levels in lungs were measured. The pulmonary expression of nuclear factor- κ B and p65 was evaluated by Western blotting.	Melatonin (10 mg/kg,i.p.) given 30 min prior to LPS administration prevented the decrease in PaO ₂ and the lung injury caused by the endotoxin. Melatonin decreased the elevated MPO activity and MDA levels after LPS. The increase of TNF- α level in pulmonary tissue given by LPS was prevented by melatonin while IL-10 levels were augmented. The nuclear localization of p65 increased markedly in LPS group and this enhancement of nuclear p65 expression was much less in the melatonin + LPS group.	[23]

Animal model	Parameters studied	Effects observed	References
LPS in streptozotocin-induced diabetic rats.	Circulating levels of TNF- α , IL-6, corticotropin-releasing hormone, ACTH and corticosterone were measured.	LPS significantly increased the serum levels of TNF- α and IL-6 in normal and diabetic rats and augmented plasma corticotropin-releasing hormone, ACTH and corticosterone. Both 0.1 and 1 mg/kg melatonin doses significantly decreased serum levels of TNF- α and IL-6. Significant inhibitory effects of melatonin (1 mg/kg) were observed on the HPA axis. Melatonin treatment helped to maintain glucose homeostasis in LPS-injected diabetic rats.	[24]

ALT: alanine aminotransferase; AST: aspartate aminotransferase; ATP: adenosine triphosphate; BCG: Bacillus Calmette Guerin; GSH: glutathione; GSH-Px: glutathione peroxidase; GSSG: oxidized glutathione; IFN: interferon; IL: interleukin; iNOS: inducible nitric oxide synthase; LPS: lipopolysaccharide; MAPK: mitogen-activated protein kinase; MDA: malondialdehyde; MPO: myeloperoxidase; NE: norepinephrine; NO: nitric oxide; SOD: superoxide dismutase; TBARS: thiobarbituric acid reactive substances; TNF: tumor necrosis factor; TUNEL: TdT-mediated dUTP nick-end labeling.

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Supplemental Table 2

Protective effects of melatonin in animal models of septic shock

Septic shock model	Parameters studied	Effects observed	References
CLP in mice following hemorrhagic shock.	Mortality rate.	Short-term melatonin administration (10 mg/kg i.p.) after hemorrhage significantly improved survival in animals subjected to septic challenge with LPS. Continuous melatonin treatment did not improve survival, as compared to vehicle-treated mice subjected to shock and CLP.	[1]
CLP in rats.	MDA and GSH levels, and MPO activity in liver, kidney, heart, lung, diaphragm, and brain. Histological examination of the same tissues.	Melatonin (10 mg/kg i.p.) given 30 min before and 6 h after the operation prevented the increase of MDA and MPO, and the decrease of GSH brought about by CLP. Melatonin also reversed the microscopic damage given by CLP.	[2]
CLP in mice.	Mitochondrial NOS activity and electron transport chain activity function in skeletal muscle of wild-type (iNOS ^{+/+}) and iNOS knockout (iNOS ^{-/-}) mice were measured. Lipid peroxidation levels, GSSG/GSH ratio, and GSH-Px and GSH reductase activities were determined.	After sepsis, iNOS ^{+/+} but no iNOS ^{-/-} mice showed an increase in mitochondrial NOS activity and NO production, a reduction in electron transport chain activity and a pronounced oxidative stress as shown by changes in lipid peroxidation levels, oxidized glutathione/reduced glutathione ratio, and glutathione peroxidase and reductase activities. Melatonin (30 mg/kg i.p.), administered 30 min before CLP and 30 min, 4 h and 8 h after CLP, counteracted all these changes in septic iNOS ^{+/+} mice.	[3]
Escherichia coli endotoxemia in rats.	Diaphragmatic fatigability and MDA level analysis as an indicator of lipid peroxidation were assessed.	Sepsis impairs diaphragmatic contractility and endurance capacity and increases diaphragmatic fatigability. Melatonin (60 mg/kg i.p.) 30 min before endotoxin inoculation to animals prevented the decrease development of diaphragm fatigue but did not affect the increase in diaphragmatic MDA levels caused by the endotoxin.	[4]
CLP in mice.	The activity of ATPase, the level of adenine nucleotides, and the ATP production were measured in mitochondria of diaphragm and hind leg skeletal muscle of iNOS ^{+/+} and iNOS ^{-/-} mice.	Melatonin (30 mg/kg i.p.), administered 30 min before CLP and 30 min, 4 h and 8 h after CLP, normalized the production of ATP in iNOS ^{+/+} mice, without affecting iNOS ^{-/-} animals	[5]

Septic shock model	Parameters studied	Effects observed	References
Candida sepsis in rats.	Plasma levels of IL-6, TNF- α , vascular cell adhesion molecule-1, and E-selectin were measured.	Daily melatonin injections (200 μ g/kg i.p.) were given a week prior to sepsis induction and were continued for 3 weeks until the end of the study. IL-6, TNF- α , vascular cell adhesion molecule-1, and E-selectin levels of septic rats were higher than those of controls. Melatonin reduced IL-6 levels and shortened time to improvement in animals with Candida sepsis.	[6]
CLP in rats.	Mortality rate, mean arterial blood pressure and vascular responses to NE, blood glucose, hepatic function index (i.e. glutamate-pyruvate transaminase, glutamate-oxaloacetate transaminase, albumin and total protein), cell toxicity index (i.e. lactate dehydrogenase) and renal function index (i.e. blood urea nitrogen and creatinine as well as plasma levels of IL-1 β and NO were measured. In addition, superoxide production by aorta, lung and livers was assessed. All organs were also histopathologically studied.	Melatonin (3 mg/kg, i.v. at 3, 6, 12 hr after CLP) significantly (a) attenuated hyporeactivity to NE and delayed hypotension, (b) reduced plasma index of hepatic and renal dysfunction, (c) diminished plasma NO and IL-1 β concentrations as well as aortic superoxide levels, (d) reduced marked infiltration of polymorphonuclear neutrophils in the lung and liver tissues, and (e) promoted the survival rate at 18 hr to twofold compared with the CLP alone group.	[7]

ATP: adenosine triphosphate; CLP: cecal ligation and puncture; GSH: glutathione; GSH-Px: glutathione peroxidase; GSSG: oxidized glutathione; IL: interleukin; iNOS: inducible nitric oxide synthase; LPS: lipopolysaccharide; MAPK: mitogen-activated protein kinase; MDA: malondialdehyde; MPO: myeloperoxidase; NE: norepinephrine; NO: nitric oxide; TNF: tumor necrosis factor.

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