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Melatonin as a Therapeutic Agent for Sepsis

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Melatonin as a therapeutic agent for sepsis

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Incidence and Current Treatment of Sepsis

With an estimated incidence exceeding 1.1 million cases per year in the United States (Moore and Moore, 2012), sepsis is the tenth leading cause of death overall in this country (Yang *et al*., 2009). As of 2005, "mortality of severe sepsis exceeds other high-profile diseases such as AIDS, venous thromboembolism, and both lung and colon cancers" (Jones, 2006). Moreover, septic shock-related mortality is greater than 40%, and sepsis itself is the leading cause of death in noncardiac Intensive Care Units, (Moore and Moore, 2012). Little progress in reducing sepsis is being made: to the contrary, between 2003 and 2007 hospitalizations for severe sepsis in the US increased by 71%, and in 2007 incurred a cost of $24.3 billion (Lagu *et al*., 2012). Mortality rates have decreased little in the past 30 years (Astiz and Rackow, 1998). As of 2005, multidrug-resistant bacteria and fungi were noted to cause about 25% of cases (Annane *et al*., 2005), increasing the importance for identifying adjuvant treatments capable of strengthening the immune system of patients.

With deficiencies in key innate immune responses, preterm infants and neonates are particularly susceptible to life-threatening infections (Strunk *et al*., 2011), including sepsis. Thereafter, the incidence and mortality of sepsis increase dramatically with age: incidence in patients over 85 years of age is 100 times that in children, with a mortality of 38.4% in this elderly age group (Angus *et al*., 2001). Thus, sepsis is primarily a disease of both the very young and the aged, with increased incidence and mortality occurring in the aged (DeGaudio *et al*., 2009; Zhou *et al*., 2010; McConnell *et al*., 2111). This may

be because the inflammatory response in the aged is exaggerated as compared to the young adult (Leong *et al*., 2010). This is in part due to the fact that inflammatory genes are expressed at high levels in aged humans and animals even in the absence of a provocative stimulus (Sharman *et al*., 2007; Bohler *et al*., 2009). This high resting level is further intensified during sepsis (Yavuz *et al*., 2007; Wang *et al*., 2010). The conditions of sepsis, severe sepsis, and septic shock constitute a continuum of increasingly severe clinical response to infection (Table 1). Patients with sepsis display manifestations of infection and inflammation, consisting of two or more of the following: increased or decreased temperature, increased or decreased leukocyte count, tachycardia, and rapid breathing (Annane *et al*., 2005). Those progressing to severe sepsis additionally develop hypoperfusion with organ dysfunction. Sepsis often progresses to septic shock, characterized by persistent vascular hypotension refractory to fluid administration and dysfunction of multiple organs, particularly heart, kidney, lung and liver. Sepsis may best be characterized as a multifactorial condition affecting multiple tissues and organs by multiple mechanisms.

Infections that induce sepsis occur most frequently in the lungs, abdomen, and urinary tract. Gram-negative bacteria are often the causative infectious agent, but increasingly sepsis also results from infection by gram-positive bacteria or fungi (van der Poll and Opal, 2008). Multiple infectious organisms may be involved as well. Fungal infections are of particular concern because they can result in substantially higher mortality (Opal *et al*., 2003).

The immunological response in septic shock is biphasic: the initial response to the infection is overwhelming inflammation; this is later followed by a period of immune

depression that may persist long after the patient has otherwise recovered from the infection.

A critical barrier to progress is that standard therapy has tended to use a select few agents each with a single mechanism of action for treating the most prominent aspects of septic shock, such as antibiotics, vasopressors, and anti-inflammatory drugs. While antibiotics may eliminate the causative agents, they do nothing to dampen the body's excessive inflammatory response that precipitates septic shock, and their creation of large quantities of pathogen fragments may actually exacerbate it. Glucocorticoids may seem attractive agents for suppressing such inflammation. Although many clinical trials using a variety of glucocorticoids have been conducted, none has conclusively proven these agents to be beneficial in treating septic shock, and in some trials, glucocorticoids actually increased mortality (Sessler, 2003). The inconclusive or detrimental nature of these outcomes is consonant with the well-known immune-suppressing properties of glucocorticoids (Löwenberg *et al*., 2007). Although the pathological hypotension associated with sepsis is induced by an increased production of nitric oxide, treatment with inhibitors of nitric oxide synthase either actually increases mortality of septic shock patients (López *et al*., 2004) or fails to improve their survival (Bakker *et al*., 2004). These examples illustrate the shortcomings of single-target treatments for a complex multi-factorial disorder.

The fact that infections are increasingly resistant to a broad range of antibiotics (Nordmann *et al*., 2011) further accentuates the urgency of finding novel approaches to sepsis that utilize the intrinsic defense mechanisms of the host.

Overview of sepsis pathology

The severity of organ dysfunction that accompanies sepsis and septic shock can be measured by evaluating the *sequential organ failure assessment* (SOFA) score. Changes in SOFA scores early in disease progression are meaningful: rapid worsening of this score in the first 48 hours led to over 50% mortality, whereas improved organ function in the first 24 hours was associated with increased survival (Vincent, 2007).

Hemostasis is a normal and beneficial host response to bacterial infection; bacteria in turn have developed a number of mechanisms for evading, subverting and dysregulating this process (Fourrier, 2012). A disorder frequently accompanying severe sepsis is disseminated intravascular coagulation (DIC). In this condition, myriad microclots are formed at locales widely distributed throughout small and mid-sized vessels. This condition seems to result in exhaustion and dysregulation of the clotting system, so that patients become overly sensitive to bleeding, particularly after major surgery. Tissue factor is the main initiator of coagulation in sepsis, and is constitutively expressed in the extravascular compartment thus initiating clotting if blood leaves the confines of the vasculature. During severe sepsis, activated monocytes and endothelial cells, along with circulating microvesicles, become sources of tissue factor. Inhibitors of the factor VIIa–tissue factor pathway in experimental studies in human beings and primates nullify the activation of this coagulation pathway (van der Poll and Opal, 2008). An important characteristic of DIC is an insufficiency of tissue factor pathway inhibitor (TFPI) (Franchini *et al*., 2006). Melatonin dose-dependently increases levels of TFPI protein in human coronary artery endothelial cells *in vitro* (Kostovski *et al*., 2011), suggesting that

its use in treating DIC in sepsis may prove useful.

There is substantial consensus that the healthy resolution of the inflammatory state—such as occurs when the body can overcome sepsis without going into shock—is not a passive process, but is under active regulatory control (Buckley *et al*., 2013).

Synthesis, Absorption and Metabolism of Melatonin

Although the pineal gland is probably the most well-known site of melatonin synthesis, this hormone is produced in many other mammalian organs, tissues and cells as well. These include the retina, the gastrointestinal tract, epithelial cells, and—of particular significance for immune function—bone marrow, thymocytes, and a variety of leukocytes, most notably monocytes, eosinophils, mast cells, T-lymphocytes, and NK cells (Hardeland *et al*., 2011). That melatonin synthesized outside of the pineal gland seems not to enter the general circulation implies that its action in extra-pineal tissues is likely to be of a local, autocrine or paracrine, nature (Hardeland *et al*., 2011).

The metabolism, pharmacokinetics and bioavailability of melatonin in humans have been reviewed (Brzezinski, 1997). Briefly, catabolism of melatonin occurs rapidly by hydroxylation, mainly in the liver; the product, 6-hydroxymelatoni, is conjugated with sulfate or glucuronic acid, and excreted in the urine. Concentrations of urinary 6-sulfatoxymelatonin (the chief metabolite of melatonin) and serum melatonin closely parallel one another (Lynch *et al*., 1975). Melatonin administered *i.v* is rapidly distributed (serum half-life, 0.5 to 5.6 minutes) and eliminated (Iguchi *et al*., 1982). The bioavailability of orally administered melatonin varies widely, but is roughly proportional to dose. In normal subjects given 80 mg of melatonin, serum melatonin concentrations

were 350 to 10,000 times higher than typical nighttime peak values 1–2½ hours later, and-remained stable over a 1½ hour period (Waldhauser *et al*., 1984). Lower oral doses of melatonin (1 to 5 mg) result in roughly proportionately reduced serum concentrations that are 10 to 100 times higher than the typical nighttime peak within one hour after ingestion, followed by a decline to base-line values in four to eight hours. Very low oral doses (0.1 to 0.3 mg) given during the day result in peak serum concentrations that are within the normal nighttime range (Dollins *et al*., 1994).

A certain proportion of melatonin administered in the diet, is able to enter the bloodstream and thence to other organs including the brain, in an intact and unconjugated form (Lahiri *et al*., 2004).

Melatonin and Aging

Preterm human infants produce little if any melatonin and require some 15 hours to clear it after exogenous administration (Merchant *et al*., 2013). Human newborns are able to synthesize melatonin, but require ~9–12 weeks to begin secreting normal circadian nighttime pulses (Kennaway *et al*., 1992). Thus optimal dosage amounts and regimens that might be contemplated for treating conditions such as sepsis in preterm and newborn infants are likely to differ from those in older patients. After adulthood, melatonin levels decline severely with age in humans and in mice (Waldhauser *et al*., 1988; Lahiri *et al*, 2004). While the full range of physiological consequences of this is unknown, it is likely that this may contribute to impairment of appropriate immune and inflammatory responses in the elderly. There is a large literature on the ability of melatonin to attenuate some of the less desirable consequences of aging (Sharman *et al*., 2011; Bondy *et al*.,

2004; Akbulut *et al*., 2008; Pierpaoli and Regelson, 1994). These effects of melatonin have been related to reversal of the age-associated deterioration of the immune system (immunosenescence) including functional decline of granulocytes, macrophages and T and B cells (Espino *et al*., 2012). Beneficial effects of melatonin have also been reported for age-associated declines in ovarian biology (Alvarez *et al*., 2012), brain functioning (Ramirez-Rodriguez *et al*., 2012), liver metabolism (Eşrefoğlu *et al*., 2012), colonic function (Pascua *et al*., 2012), cardiac effectiveness (Foreman *et al*., 2011), and increased pancreatic insulin resistance (Cuesta *et al*., 2013). Many of these effects have been attributed to an improvement of immune function and a regulation of inflammatory responses.

Melatonin treatment of aged animals can largely reverse many of the changes in gene expression that characterize aging. This is especially true of genes related to the immune system and to inflammation, which are generally elevated with age and hyper-responsive to inflammatory stimuli (Sharman *et al*., 2007, 2008). These changes both in basal mRNA levels and in their reactivity may be the cause underlying many reported anti-aging properties of melatonin. The relevance of these findings to the treatment of sepsis is that the inflammatory response found with sepsis is markedly heightened with aging (Turnbull *et al*., 2009).

Melatonin, the Immune System and Inflammatory Responses

Melatonin is synthesized in many types of immune cells; moreover, two plasma-membrane melatonin receptors are found to reside on immune cells, and melatonin has been determined to influence many cellular as well as whole animal immune functions

(Carrillo-Vico *et al*., 2013).

Cytokine and chemokine signaling networks are altered in elderly patients, and tend to favor a type 2 cytokine response (largely humoral) over a type 1 (largely cellular) response (Opal *et al*., 2005). It is the former which can lead to generalized inflammation while the latter represents a more selective and localized immune response. Thus this imbalance can lead to excessive production of pro-inflammatory factors (Opal *et al*., 2005). A failure of the type-1 arm of the immune system and an overactive type-2 arm is implicated in a wide variety of chronic illnesses. It has been proposed that development of new drugs that specifically regulate the balance of these two kinds of response activity may pave the way for novel therapeutic interventions in sepsis (Matsukawa *et al*., 2001). Melatonin may be such an agent (Petrovsky and Harrison, 1997), since it can simultaneously up-regulate a type-1 immune response while down-regulating the type-2 response which involves interleukin (IL)-4, IL-10 production and splenocyte proliferation (Santello *et al*., 2008).

Lipopolysaccharide treatment is often used as a means of provoking acute inflammatory responses in experimental animals (endotoxemia). However, low levels of LPS have also been used to model sepsis *in vitro* (Lowes *et al*., 2011). Responses evoked in this manner have frequently been found to be attenuated by prior treatment with melatonin both in whole animals (Zhong *et al*., 2009; Fugundes *et al*., 2010) and in isolated cell systems (~~**Nath *et al*., 2012;** ~~Lowe *et al*., 2011). This suppression by melatonin may be related to reduced expression of several inflammatory genes, since melatonin is able to reverse the heightened expression of both the basal and LPS-induced levels of expression of

inflammatory cytokines found in aging brain (Yavuz *et al*., 2007; Sharman *et al*., 2007; Perreau *et al*., 2007~~; Kim, 2011~~). That the protective effect of melatonin is systemic, is well illustrated by the finding that it can markedly reduce the lethality of LPS (Requintina and Oxenkrug, 2003). Following intraperitoneal injection of LPS into rats, there was an increase in the TBARS levels, and in apoptotic cell death. Concurrent administration of melatonin prevented these changes and led to increased activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase (Ozdemir *et al*., 2007).

Melatonin and sepsis

Melatonin may be a means by which the severity of sepsis can be mitigated and there are several mechanisms by which this might occur. Melatonin can act to regulate inflammatory processes. The timing of these effects suggests that melatonin can promote the early phases of an immune response and attenuate the later stages of inflammation. Thus an effective immune defence is initially fostered by melatonin and this is followed by prevention of chronic and harmful inflammatory events (Radogna *et al*., 2010). The later stages of melatonin action involve reducing induction of IL-6, cyclooxygenase-2, tumor necrosis factor (TNF)-α and inducible nitric oxide synthase (iNOS) (Deng *et al*., 2006; Yavuz *et al*., 2007). Thus, melatonin is capable of sophisticated regulatory changes rather than acting merely as a broad anti-inflammatory or anti-oxidant agent. In addition, many oxidant responses associated with sepsis such as depletion of reduced glutathione levels and elevated lipid peroxidative activity, are reversed by melatonin treatment (Şener *et al*., 2005). In view of the very low levels of free melatonin

encountered within cells, such changes are likely to be mediated by melatonin either acting through its receptors or effecting alterations in gene expression (Sharman *et al*., 2007; Perreau *et al*., 2007).

Animal models of sepsis

A wealth of studies in animals suggests the value of melatonin in treating sepsis. The survival rates found in several different animal models of sepsis benefit from melatonin treatment (Wichman *et al*., 1996; Reynolds *et al*., 2003; Zhang *et al*., 2012). Mice subjected to a combination of hemorrhagic shock followed by septic challenge exhibited reduced mortality when treated for a short time with melatonin (Wichmann *et al*., 1996). Some aspects of sepsis in specific organs are described, together with an outline of the benefits of melatonin treatment to organ systems impacted by experimental sepsis.

Gastrointestinal tract: The leakage of digestive enzymes from the intestinal lumen into surrounding tissue is commonly associated with both septic and hemorrhagic shock. According to the autodigestive hypothesis for the initiation of the systemic inflammatory response, errant digestive enzymes not only attack and damage intestinal tissue, but are also responsible for damage to tissues in other organs such as heart and lung, thus contributing to multi-organ failure (Schmid-Schönbein, 2008). Inhibiting these enzymes in the intestinal lumen can substantially increase survival of animals subjected to shock (Delano *et al.,* 2013).

Although the pineal may be more widely known as a source of melatonin, the

gastrointestinal tract is in fact the major site of melatonin synthesis in the body (Bubenik, 2002). Decreased gastro-intestinal motility is characteristic of sepsis (Königsrainer *et al*., 2011) and melatonin is able to completely reverse the arrest of gastro-intestinal motility that is effected by LPS treatment (De Filippis *et al*., 2008).

Liver: The liver is one of the first organs impacted by sepsis. Sepsis alters hepatic transcription of genes for adaptive acute phase proteins, represses transcription of genes for proteins involved in phase I and II metabolism and transport, and increases levels of transaminases and alkaline phosphatase (Bauer *et al*., 2013). Intracellularly, *i.v.* endotoxin injection in **rats**~~**mice**~~ increased liver ~~**mitochondrial**~~ **iNOS activity and** NO levels**, accompanied by increases in tissue levels of alkaline phosphatase and the transaminase alanine aminotransferase (ALT)** ~~**and decreased complex IV activity**~~; *i.p*. injection of melatonin reversed these effects (Escames *et al*., 200**6**~~**3**~~). In a rat model of acute sepsis, melatonin ameliorated the liver damage indicated by elevated levels of ~~**the transaminase**~~ ALT, **and** decreased mitochondrial complex IV activity and the ATP:oxygen ratio (Lowes *et al*., 2013).

Hepatic ischemia/reperfusion (I/R) induces many of the same indicators of inflammatory damage as does sepsis. In the rat, during hepatic I/R, levels of endothelin-1 (ET-1) and its receptor, ET(B) mRNA, were elevated but attenuated by melatonin. mRNA levels of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), heme oxygenase-1 (HO-1) and TNF-α were all elevated after I/R. Melatonin augmented the increased expression of eNOS mRNA, whereas it reduced the increase in iNOS mRNA

and TNF-α. (Park *et al*., 2007). In a later study, melatonin was found to lower the expression of numerous pro-inflammatory cytokines and pro-apoptotic genes that were raised by I/R, and to improve liver function (Kireev *et al*., 2012). These studies suggested an overall anti-inflammatory effect.

Septic shock and hemorrhagic shock also inflict rather similar types of damage to the liver. Damage by both is mediated by a reduction in akt phosphorylation, by increases in iNOS and HO-1, and accompanied by an increase in plasma myeloperoxidase. In the case of hemorrhagic shock in rats, melatonin can normalize liver akt phosphorylation, plasma myeloperoxidase, and reduce liver damage (Hsu *et al*., 2012).

Heart: Cardiac dysfunction occurs frequently in septic patients. It is reported that the left ventricular ejection fraction (LVEF) in hemodynamically unstable septic shock patients was depressed and their stroke volumes were severely reduced; one in six had an LVEF of less than 30% (Rudiger and Singer, 2007). Moreover, occurrence of myocardial depression in septic patients tends to be associated with increased mortality (Court *et al*., 2002).

Repeated administration of melatonin to rats following cecal ligation and puncture (CLP) improved heart mitochondrial function and increased survival at 48 hours post CLP (Zhang *et al*., 2013). Melatonin (30 mg/kg, *i.p*.) was administered at 3 hours post CLP, and then injected subcutaneously every 3 hours thereafter up to the 24 hour point. After 48 hours, melatonin restored the substantial reductions in base excess and lactate induced by CLP, and increased the proportion of animals surviving from 32% to 57%. Melatonin also restored the more modest reductions in CLP-induced left ventricular ejection fraction

and cytochrome c oxidase (COX) activity. In contrast to the many animal studies that begin melatonin treatment prior to injury, in this study, melatonin was administered starting 3 hours after CLP and after onset of symptoms. Consequently, this design is more relevant to clinical applications and provides stronger evidence that melatonin treatment would be of real benefit in the ICU. In a study in mice generally similar to the above, melatonin was also found to improve heart mitochondrial function following CLP: CLP-induced reductions in COX levels were restored, elevated iNOS levels were normalized, as were indices of lipid peroxidation and protein oxidation (Ortiz *et al*, 2013). These effects of melatonin were independent of nNOS gene knockout.

Lung: Acute lung injury commonly develops during sepsis, with nearly half of patients in one observational study developing this condition (Iscimen *et al*., 2008). In an animal model of acute septic lung injury induced by LPS injection, administration of melatonin was shown to have beneficial effects in rats (Shang *et al.*, 2009). In this study, melatonin reduced the LPS-induced pulmonary leukocyte infiltration, elevated levels of tissue malondialdehyde ~~(MDA)~~, myeloperoxidase, and TNF-α, while it augmented levels of the typically anti-inflammatory cytokine IL-10. In an earlier study, *i.v*. endotoxin injection in **rats**~~**mice**~~ increased **lung**~~**liver**~~ mitochondrial NO levels, mitochondrial NOS activity and decreased activities of complexes I and IV; *i.p*. injection of melatonin reversed all these effects (Escames *et al*., 2003). Melatonin administration (*i.p*.) also has produced beneficial effects in lungs of rats in which sepsis had been induced by CLP; melatonin reduced the sepsis-elevated levels of malondialdehyde and improved the sepsis-induced

pulmonary structural degeneration, vasocongestion and edema observed (Şener *et al*., 2005).

Vasculature: Melatonin has been reported to prevent endotoxin-induced circulatory failure in rats (Wu *et al*., 2001). Such circulatory failure was attributed to inhibition of (i) the release of TNF-α in plasma, (ii) the expression of NOS II in liver, and (iii) the production of superoxide in the aorta. Another study suggests that melatonin may improve outcomes of sepsis due to fungal infections (Yavuz *et al*., 2007). Rats were first immunosuppressed with cyclophosphamide and then received an *i.v*. injection of *Candida albicans*. Subsequent daily *i.p*. injections of 200 μg/kg melatonin reduced serum levels of the proinflammatory cytokine IL-6 after 15 days, and reduced the time required for *Candida* to be cleared from the blood stream by 15%.

Nerve and muscle: Critical illness polyneuropathy and myopathy commonly occur after the onset of severe sepsis (Latronico and Bolton, 2011). Melatonin, particularly in combination with oxytocin is effective in reducing the electromyographical, inflammatory, and oxidative dysregulation associated with critical illness polyneuropathy occurring after induction of sepsis in the CLP rat model (Erbaş *et al*., 2013).

Central nervous system: The brain plays a key role in sepsis, since it acts as both a mediator of the immune response and a target for the pathologic process (Zampieri *et al*., 2011; Gofton and Young, 2012). Septic encephalopathy is characterized by alteration of

consciousness, occurrence of seizures or focal neurological signs and involves an ischemic process, secondary to impairment of cerebral blood flow and neuroinflammatory events including endothelial activation, alteration of the blood-brain barrier and passage of neurotoxic mediators (Adam *et al*., 2013). The mortality rate of septic patients with altered mental status was 49% compared with a rate of 26% in septic patients with no neurological symptoms (Sprung *et al*., 1990). While no clinical study has focused specifically on melatonin and septic encephalopathy, work on septic shock in mice has demonstrated melatonin's protective effect to extend to several organs including the brain (Carrillo-Vico *et al*., 2005). A study demonstrating the beneficial effects of melatonin upon an animal model for neonatal encephalopathy (Robertson *et al*., 2013) is also pertinent.

The vast majority of studies of the effects of melatonin on sepsis have used murine models, undertaken with the implicit assumption that responses in septic humans and rodents are largely similar. Recently, a comparison of 4918 human genes including those responsive to an *i.v*. LPS injection demonstrated that their changes are uncorrelated with changes in the corresponding orthologs in mice treated similarly (Seok *et al*., 2013). On the other hand, substantial similarity of gene expression patterns (and some useful disparity) was found between mice injected *i.p*. with human feces and severely septic pediatric patients (Lambeck *et al*., 2012). Thus, while the latter study gives confidence that rodents and humans respond to live-bacterial infection in substantially and usefully similar ways, the results of the former (produced under more artificial conditions) imply that caution may be required in applying results of animal studies that utilize LPS, to the treatment of sepsis in humans.

Human clinical trials

Melatonin status is altered under conditions of sepsis. Urinary melatonin excretion of septic patients in a state of shock was higher than that of septic patients not in septic shock and of those of non-septic patients (Bagci *et al*., 2011). To date, there has only been one human study relating to the utility of melatonin in treatment of sepsis—and that ~~was~~ in neonates. Treatment with melatonin may be of particular relevance in this group of patients, since human infants do not begin producing melatonin normally until 2-4 months of age. In a clinical trial of melatonin, the drug was found effective in reducing the mortality of neonatal sepsis and at 48 hours was associated with statistically-significant improvements in counts of total white blood cells, absolute neutrophils and platelets, and levels of C reactive protein (Gitto *et al*., 2001). Numerous laboratory studies provide a rationale for these results: in addition to the above parameters, a wide variety of inflammatory factors are increased in neonatal sepsis (Sugitharini *et al*., 2013) and under conditions in which these factors are similarly increased, melatonin has been reported to normalize them in most cases (Table 3). Moreover, other clinical trials suggest anti-bacterial properties of melatonin. These include acceleration of healing of ulcers associated with *Helicobacter pylori* (Celinski *et al*., 2011) and mitigation of ulcerative colitis associated with several pro-inflammatory bacterial species (Wang *et al*., 2007).

Since sepsis involves an excessive inflammatory response leading to massive outpouring of cytokines, other clinical trials reporting anti-inflammatory benefits of melatonin are

also relevant to sepsis (Cichoz-Lach *et al*., 2010; Ochoa *et al*., 2011). Therefore it is appropriate to consider reports of melatonin treatment of related conditions. In ulcerative colitis patients, melatonin treatment maintained normal levels of C-reactive protein (between 3.0 and 4.2 mg/dl), compared to the elevated levels of 13.1 mg/dl in the untreated patients (Chojnacki *et al*., 2011). This suggests the possibility that melatonin may be capable of reducing the elevated C-reactive protein levels associated with sepsis. Burn injuries can often lead to compromise of protective epidermal barriers and generalized sepsis. A relatively low dose of melatonin (3 mg/d) is sufficient to reduce mortality, shorten healing time and lower the incidence and distribution of invading bacterial species in patients suffering from severe burns (Sahib *et al*., 2010).
Serum troponin levels are elevated in critically ill septic patients, and increased levels are associated with lower cardiac stroke ejection fractions and increased mortality (Rudiger, 2007). While perioperative administration of 60 mg of melatonin *i.v*. to patients undergoing major abdominal aortic surgery failed to reduce the elevated troponin levels associated with this procedure (Kücükakin *et al*., 2008), similarly elevated troponin levels in a rat model of cardiac ischemia-reperfusion were substantially reduced by melatonin administered 10 mg/kg, *i.p*. 30 minutes prior to injury (Acikel *et al*., 2003).

Sepsis and the circadian cycle

The circadian rhythm of melatonin secretion is altered in the early stages of sepsis in human ICU patients, with the acrophase of melatonin secretion being shifted from 2 am to 6 pm in the non-septic and septic patients, respectively (Li *et al*., 2013). In these

patients, peak levels of plasma TNF-α and IL-6 occurred in concordance with peak melatonin secretion, while mRNA levels of the circadian clock genes *cry-1* and *per-2* were suppressed. It is also noteworthy that sleep deprivation after septic insult increases mortality (Friese *et al*., 2009). Sleep deprivation experienced in the ICU setting during sepsis may thus be deleterious. Thus another potential beneficial aspect of melatonin is its ability to regulate and restore normal sleep patterns.

Hypotension associated with sepsis

Septic shock is characterized by hypotension and vascular hyporeactivity to contractile agents. Melatonin can restore endothelium-derived constricting factor signaling and consequent regulation of the inner diameter in the rat femoral artery after inhibition of nitric oxide-based vasodilation (Paulis *et al*., 2010). It can also prevent lipopolysaccharide-induced vascular hyporeactivity in rat (d'Emmanuelle *et al*., 2004). By this means melatonin can increase vascular perfusion and reverse hypotension. Melatonin can also reverse the refractory hypotension associated with multiple organ dysfunction syndrome of septic shock (Wu *et al*., 2008). Remarkably, melatonin is also able to reduce blood pressure in essential hypertension (Cagnacci *et al*., 2005). Thus this moiety is neither intrinsically hypo- nor hypertensive but appears able to regulate blood pressure in a bidirectional manner.

Mechanisms Underlying Melatonin's Actions

Although a broad range of protective effects of melatonin have been described, it is likely that there is a much more limited number of key mechanisms that underlie these effects.

Whether these are mediated by specific receptors or can be attributed to direct effects of melatonin remains uncertain (Reiter *et al*., 2007). Studies in isolated systems need to take account of physiological concentrations of free melatonin which are very low**;** this is the case especially in tissues other than serum (Lahiri *et al*., 2004, Table 2) and gastrointestinal tract (Bubenik, 2001), in which melatonin concentrations can be substantial. Melatonin is reported to possess direct antimicrobial activity but this is at concentrations much higher than those found in intact animals (Tekbas *et al*., 2008). This intrinsic property against multidrug-resistant gram-positive and gram-negative bacteria would be very relevant to sepsis (Srinivasan *et al*., 2012), but it is more likely that antibiotic effects are mediated by melatonin's promotion of immune targeting.

Receptor-mediated mechanisms

Three plasma membrane receptors for melatonin have been identified—the G-protein coupled receptors MT1, MT2, and the quinone dehydrogenase enzyme NQO2.

The anti-inflammatory properties of melatonin are blocked by luzindole, a non-specific MT1 and MT2 receptor antagonist (Cevik *et al*., 2005), suggesting an involvement of receptors in this protective effect. In particular, both the MT1 receptor and its G-protein target G_{16} are found in hematopoietic cells, implying that melatonin may modulate hematopoietic growth and immune function; cytokine production and STAT3 phosphorylation resulting from MT1 activation by melatonin in Jurkat T cells supports this notion (Chan and Wong, 2013). It has been proposed that MT1 receptors are key in facilitating some protective roles of melatonin (Renzi *et al*., 2011). Involvement of the

MT2 receptor in immune function has been shown more directly in mice. Melatonin was found to enhance splenocyte proliferation and IgG antibody response in these animals; while these effects were unchanged by knockout of the MT1 receptor gene, they were attenuated by administration of the MT2 receptor antagonist luzindole (Drazen and Nelson, 2001). However, in view of the incomplete characterization of these receptor types, much remains unresolved.

The third of melatonin's plasma-membrane receptors, MT3, is the enzyme NRH:quinone oxidoreductase, NQO2. Knockout of the NQO2 gene in mice lowered peripheral blood B cell count, altered the homing behavior of those B cells that were present, decreased the germinal center response, and impaired antibody responses (Iskander *et al*, 2006). In addition, these mice exhibit decreased expression of NF-κB, suppression of its activation, and altered chemokines and chemokine receptors. These changes were suggested to lead to the deficiency in B-cell numbers. Alterations in B cell homing behavior and impaired humoral immune response also were observed in this study (Iskander *et al*., 2006). Because of the very low levels of tissue melatonin, it is more likely that, rather than acting directly, its ability to regulate immune function and act as an antioxidant is based on a cascade of magnification. As indicated above, this is likely achieved by way of activation of specific receptors, leading to inhibition or stimulation of transcription factors, and thence to altered expression of crucial genes relevant to immune function and antioxidant enzymes. There is considerable evidence for such a postulated trajectory. This comprises demonstration of inhibition by melatonin of activation of pro-inflammatory transcription factors such as nuclear factor kappa B (Lowes *et al*., 2011) and inflammatory kinases such as JNK (De Filippis *et al*., 2008) in LPS-modeling of

sepsis. This pathway can lead to down-regulation of genes associated with inflammation and up-regulation of genes for antioxidant enzymes (Sharman *et al*., 2007, 2008; Garcia *et al*., 2010; Laothong *et al*., 2010),

Antioxidant and anti-inflammatory mechanisms

The protective mechanisms against sepsis appear to involve both antioxidant (Li Volti *et al*., 2012) and anti-inflammatory properties of melatonin (Erbas *et al*., 2013). Other potential mechanisms may include the ability of melatonin to increase plasma albumin levels (El-Missiry *et al*., 2007; Oz and Ilhan, 2006). Low albumin is a mortality risk factor for elderly septic patients. Melatonin may also slow the translocation of bacteria between various organ systems, retarding the development of generalized infection (Akan *et al*., 2008).

Bacterially-generated LPS induces iNOS synthesis in the host species, resulting in production of large amounts of NO that are toxic not only to the invading pathogens, but also to the host cells by inactivation of enzymes, leading to cell death (McCann *et al*., 1998). Melatonin significantly attenuates the LPS-induced upregulation of both cyclo-oxygenase and iNOS in RAW264.7 macrophages (Xia *et al*., 2012).

Melatonin treatment blunts the induction of mitochondrial iNOS isoforms after sepsis and thus protects against the ensuing impaired mitochondrial function. Since heart mitochondria from $iNOS^{-/-}$ mice are unaffected during sepsis, the induction of mitochondrial iNOS is associated with sepsis-related mitochondrial dysfunction (Escames *et al*., 2007). Similarly, in muscle mitochondria from $iNOS^{-/-}$ mice, ATP production was unaffected by CLP sepsis, and melatonin restored the reduced production

of ATP induced by sepsis in iNOS$^{+/+}$ animals (López *et al*., 2006).

Myeloperoxidase is a key participant in the microbicidal *oxidative burst* generated by neutrophils upon encountering invading pathogenic organisms. Its generation of reactive oxidative species is an important contributor to the inflammatory response to infection, but this response must soon be suppressed in order to avoid causing the excessive oxidative damage to the host associated with severe sepsis and septic shock. The importance of this suppression may be inferred from the increased survival of myeloperoxidase-null mice following sepsis-induced lung injury (Brovkovych *et al*., 2008). Melatonin at physiologically and pharmacologically meaningful concentrations, inhibits myeloperoxidase (Galijasevic *et al*., 2008); hence its inhibitory action may contribute to the process of resolving inflammation following sepsis.

Conclusions

A distinctive feature of melatonin is the subtlety of its actions. It is not a broad-spectrum anti-inflammatory and immune-quenching agent. Neither is it a potent non-specific anti-oxidant. Its low content within cells suggests actions through receptor linked transcription factors followed by selective modulation of gene expression. This property allows a more refined approach to the treatment of sepsis.

The mortality associated with sepsis is unacceptably high and despite substantial effort, survival has improved little over the past few decades. During this time, numerous studies have shown melatonin to have beneficial effects in sepsis and in sepsis-related conditions. These studies have been conducted utilizing a wide variety of systems: *in*

vitro cell cultures, tissues, and whole animals exposed to endotoxin or to live bacteria. These studies reported many kinds of measurements: indicators of immune function, inflammation, and oxidative damage, changes in cell and tissue morphology, changes in animal gene expression patterns, and in animal survival. This large body of evidence derived both from human and animal studies for over a decade, strongly suggests that melatonin may have significant utility in the treatment of sepsis. No significant toxicity due to melatonin—even at high pharmaceutical dosages—has accompanied the uniformly positive effects reported; moreover, its cost is minimal (Bondy and Sharman, 2010). The results of these many laboratory studies have been confirmed by the results of the single limited clinical trial undertaken to date, in human newborns. It is indeed surprising that so few human clinical trials have been conducted.

Taken as a whole, results of these studies suggest that, while in no sense should melatonin be considered as a cure or primary treatment for sepsis, incorporation of its administration in standard treatment protocols offers the possibility both of lowering the mortality due to sepsis and of reducing the long term damage often inflicted on survivors' immune systems.

The intent of this review is to draw attention to this potential in the hope that more appropriate human trials will be performed. The potential benefits of melatonin therapy are balanced by minimal risks, and it would be tragic if its utility were overlooked for insufficient transmission of information.

In view of the considerable body of evidence supporting the potential value of melatonin, it may be asked why the approach has not received more attention. Some of the answers to this may involve the inexpensiveness and ready availability of melatonin, resulting in a

lack of opportunity for commercial development and proprietary marketing by the pharmaceutical industry. However, this industry is currently developing melatonin analogs which activate specific melatonin receptors. Nevertheless, despite the need for better understanding of the mechanisms underlying this hormone's effects during sepsis, melatonin is deserving of more detailed examination in a clinical situation than it has hitherto received.