

Melatonin as a potential therapy for sepsis: a phase I dose escalation study and an ex vivo whole blood model under conditions of sepsis

Abstract: Sepsis is a massive inflammatory response mediated by infection, characterized by oxidative stress, release of cytokines, and mitochondrial dysfunction. Melatonin accumulates in mitochondria, and both it and its metabolites have potent antioxidant and anti-inflammatory activities and may be useful in sepsis. We undertook a phase I dose escalation study in healthy volunteers to assess the tolerability and pharmacokinetics of 20, 30, 50, and 100 mg oral doses of melatonin. In addition, we developed an ex vivo whole blood model under conditions mimicking sepsis to determine the bioactivity of melatonin and the major metabolite 6-hydroxymelatonin at relevant concentrations. For the phase I trial, oral melatonin was given to five subjects in each dose cohort (n = 20). Blood and urine were collected for measurement of melatonin and 6-hydroxymelatonin, and symptoms and physiological measures were assessed. Validated sleep scales were completed. No adverse effects after oral melatonin, other than mild transient drowsiness with no effects on sleeping patterns, were seen, and no symptoms were reported. Melatonin was rapidly cleared at all doses with a median [range] elimination half-life of 51.7 [29.5–63.2] min across all doses. There was considerable variability in maximum melatonin levels within each dose cohort, but 6-hydroxymelatonin sulfate levels were less variable and remained stable for several hours. For the ex vivo study, blood from 20 volunteers was treated with lipopolysaccharide and peptidoglycan plus a range of concentrations of melatonin/6-hydroxymelatonin. Both melatonin and 6-hydroxymelatonin had beneficial effects on sepsis-induced mitochondrial dysfunction, oxidative stress, and cytokine responses at concentrations similar to those achieved in vivo.

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Key words: 6-hydroxymelatonin, cytokines, melatonin, phase I clinical trial, sepsis

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Received February 27, 2014;
Accepted March 14, 2014.

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Introduction

Around 37,000 people die from sepsis in the UK each year and as many as 8 million every year worldwide. Although the Surviving Sepsis Campaign, a performance improvement effort by hospitals across Europe, South America, and the United States, has improved outcomes, the mortality rate remains at 31% overall and >70% in those patients who go on to develop sepsis-induced multiple organ failure [1].

Oxidative stress in patients with sepsis has been consistently described over the last 20 yr [2]. Mitochondrial dysfunction initiated by oxidative stress drives inflammation and is generally accepted as playing a major role in sepsis-induced organ failure [3]. It has been recognized that exogenous antioxidants may be useful in sepsis [2], and more recently, the potential for antioxidants acting specifically in mitochondria has been highlighted [4, 5]. We showed previously that antioxidants targeted to mitochondria, including melatonin, reduced organ damage in a rat model of sepsis [6, 7]. Exogenous melatonin has potent antioxidant activity [8, 9], and it accumulates throughout cells,

particularly in mitochondria [10]. Metabolites of melatonin also have antioxidant activity, and products from the reactions with oxidant species are also antioxidants [9, 11, 12].

In vitro models of sepsis show that melatonin and its major hydroxylated metabolite, 6-hydroxymelatonin, are both effective at reducing the levels of key inflammatory cytokines, oxidative stress, and mitochondrial dysfunction [8, 9]. In rat models of sepsis, melatonin reduces oxidative damage and organ dysfunction and also decreases mortality [7, 13–15]. The dose needed for antioxidant action is thought to be considerably higher than that given for modulation of the sleep–wake cycle, but the actual dose required in man is unclear, particularly because the major bioactive effects of oral melatonin in the context of inflammation are likely to be mediated primarily by metabolite levels.

We undertook a phase I dose escalation study in healthy volunteers using various doses of melatonin. We also developed an ex vivo whole blood/leukocyte model under conditions mimicking sepsis to determine the relative bioactivity of melatonin and its major hydroxylated metabolite,

6-hydroxymelatonin, at concentrations achieved in vivo after oral dosing.

Materials and methods

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich, Poole, Dorset, UK, and were of the highest grade available.

Clinical trial

The trial was registered at ClinicalTrials.gov (NCT01724424) and ISCTRN (17088991).

After obtaining Clinical Trial Authorisation for a Clinical Trial of an Investigative Medical Product (CTIMP) from the Medicine and Healthcare Regulatory Authority (MHRA), plus Research Ethics and NHS Research and Development approval, healthy male volunteers aged 18–30 were invited to take part by advertising. Exclusion criteria were smoking, body weight above 100 kg, any regular medication or chronic health condition, or lack of consent. After obtaining written informed consent, the study visit took place in a side room of the Intensive Care Unit at Aberdeen Royal Infirmary, Aberdeen, UK. All subjects fasted for 12 hr before the study start at 8:00 hr and were requested to abstain from alcohol for 24 hr preceding the study visit. EKG electrodes, a noninvasive blood pressure cuff, a pulse oximeter, and an intravenous cannula were sited, and a basal blood sample was taken. Basal measurements of heart rate, oxygen saturation, EKG parameters, and blood pressure were recorded. Subjects then took melatonin (*N*-acetyl-5-methoxytryptamine or *N*-[2-(5-methoxy-1H-indol-3-yl)ethyl]acetamide) as 10 mg capsules (prepared by Nu-Pharm Laboratories Ltd., Deeside, Cheshire, UK, using melatonin manufactured and supplied by Flamma s.p.a., Bergamo, Italy).

A standard open-label dose escalation study design was used with sequential assignment of cohorts of five subjects each to doses of 20, 30, 50, and 100 mg. Venous blood samples were taken after 10, 30, 60, and 120 min and 3, 4, and 6 hr for melatonin and 6-hydroxymelatonin measurement, respectively. Routine biochemistry/hematology analyses were conducted at baseline and 6 hr, and a further blood sample for melatonin and 6-hydroxymelatonin was taken at 24 hr. Urine was also collected for 6 hr. Subjects ate a standardized sandwich lunch exactly 3 hr after the melatonin capsules, and water was allowed ad libitum. Subjects were questioned at 30-min intervals about any symptoms and whether they felt drowsy.

Subjects completed validated sleep scales about their sleep the day before the study visit, the study visit day itself, and 1 wk later. The Verran and Snyder-Halpern sleep scale uses 100-mm visual analogue scales to record three domains: sleep disturbance, sleep efficiency, and sleep supplementation [16].

A safety algorithm was designed which prospectively defined gradings for each biochemical and physiological parameter with clear stopping criteria. After each dose cohort, an independent Data Monitoring Committee (DMC) comprising a biochemist and two intensive care consultants, one local and one external, assessed the data

and decided whether the investigators should continue to the next dose cohort.

Measurement of melatonin and 6-hydroxymelatonin

Blood samples were stored at 4°C until clotted and then immediately centrifuged, and the serum was frozen at –80°C until assay. Melatonin was measured using a Thermo Surveyor-TSQ Quantum liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Thermo Scientific, Hemel Hempstead, UK), which was set up and validated in-house. Sample aliquots (100 μ L) were spiked with 10 μ L of 60 ng/mL *N*-acetyltryptamine (internal standard), and plasma proteins were precipitated by the addition of 100 μ L of 1.5% (w/v) sulfosalicylic acid. After vortex mixing, 500 μ L of water was added and the samples were centrifuged at 4000 g for 10 min at 4°C. A 5- μ L aliquot of the supernatant was injected onto the LC-MS/MS system equipped with a 150 \times 2.1 mm, ACE 3 μ C₁₈ column (Hichrom Ltd, Reading, UK) maintained at 50°C. Samples were maintained at 4°C prior to injection. Elution was achieved under isocratic conditions with a mobile phase of water/methanol (50/50) containing 0.1% (v/v) formic acid at a flow rate of 200 μ L/min. Electrospray ionization was operated in positive ion mode with conditions optimized by flow injection analysis of the individual compounds: spray voltage, 4000 V; sheath gas pressure, 10 (arbitrary units); auxiliary gas pressure, 5 (arbitrary units); capillary temperature, 375°C; skimmer offset, –10V; collision pressure, 1.6 mTorr; and collision energy, 13 V. Tandem mass spectrometry was performed in single reaction monitoring (SRM) mode using the transition *m/z* 233–*m/z* 174 for melatonin and *m/z* 203–*m/z* 144 for *N*-acetyltryptamine. Under these conditions, melatonin eluted at 3.1 min and *N*-acetyltryptamine eluted at 3.5 min. Quantitation was based on an internal standard method using multilevel calibration curves (0.5–100 ng/mL melatonin) prepared in 5% bovine serum albumin with concentration plotted against the peak area ratio of the analyte to the internal standard. Weighted least squares regression analysis was carried out using Thermo Xcalibur software v.2.07 SP1 (Thermo Scientific, Hemel Hempstead, UK).

The metabolite 6-hydroxymelatonin was measured in serum and urine as the sulfated form using commercially available enzyme immunoassays (Cusabio Biotech, Hubei Province, China, and US Biologicals, Salem, MA, USA, respectively).

Ex vivo study

After obtaining University of Aberdeen College of Life Sciences and Medicine Ethics Review Board approval, a second group of 20 healthy male volunteers was invited to take part by advertising. Exclusion criteria were again smoking, body weight above 100 kg, any regular medication or chronic health condition, or lack of consent.

A single venous blood sample was collected at 9:00 hr into lithium-heparin tube and used immediately for whole blood experiments or allowed to cool to room temperature before isolating leukocytes. A sample of blood was also collected into an EDTA tube for hemoglobin and full cell

count determination. Leukocytes were isolated by sedimenting blood through 4% succinylated gelatine ('Gelofusine', B. Braun Medical Ltd., Sheffield, UK). Briefly, whole blood was centrifuged at 750 g for 10 min and the plasma removed and replaced by equal volumes of Gelofusine and RPMI 1640 medium (GE Healthcare, Chalfont St Giles, UK) supplemented with 10% fetal calf serum. The tube was then placed at 37°C for 30 min at a slight angle to allow erythrocytes to sediment. The leukocyte-rich upper layer was then aspirated and centrifuged at 500 g for 10 min, and the remaining erythrocytes were removed by hypotonic shock. The leukocyte pellet was then washed and resuspended in medium.

For experimentation, whole blood or isolated leukocytes were placed into 24- or 96-well plates (see experimental detail below) in the presence of 2 µg/mL lipopolysaccharide (LPS, from *E. coli* strain 055:B5) plus 20 µg/mL peptidoglycan (PepG, from *S. aureus* strain 6571), prepared as described previously [17]. In addition, blood or cells were also exposed to solvent control or either melatonin or 6-hydroxymelatonin (Sigma-Aldrich Ltd) dissolved in ethanol (<1% v/v) at 0, 0.01, 0.1, 1, 10, 100, or 1000 ng/mL for 18 hr at 37°C in a humidified atmosphere of 95% air/5% CO₂. The amount of LPS and PepG used in this study was determined based on optimal release of interleukin (IL)-6 in preliminary experiments (data not shown).

Cytokine and lipid hydroperoxide levels

Whole blood was treated as above in 24-well plates. Levels of IL-6 and IL-10 were measured in plasma using commercially available enzyme immunoassay kits (R&D Systems Europe Ltd, Oxford, UK). Plasma lipid hydroperoxide (LPO) levels were measured using a commercially available spectrophotometric kit (Cambridge Bioscience Ltd, Cambs, UK) after extracting LPO into chloroform. Precision data in our hands were as follows: intra-assay—IL-6 = 1.4%, IL-10 = 1.9%, LPO = 1.7%; interassay—IL-6 = 6.3%, IL-10 = 6.2%; LPO = 2.0%.

Respiratory burst

Whole blood (200 µL) was added to 800 µL phosphate-buffered saline (PBS, 1.37 M NaCl, 27 mM KCl, 95 mM NaH₂PO₄, 15 mM KH₂PO₄, pH 7.4) containing 5 mM glucose and treated immediately as described previously. Samples (100 µL) were transferred to 96 wells, and basal luminescence was measured before the addition of sodium luminol using an automated system (1.25 mM final concentration). Luminescence was measured every minute until a plateau was reached. Results were normalized to total monocyte/neutrophil number and hemoglobin content.

Mitochondrial superoxide production

Mitochondrial superoxide production was determined in intact leukocytes in 96-well plates following cell treatments as described previously [18]. Briefly, cells were washed twice with PBS before being loaded with 5 µM MitoSox™ (Life Technologies, Paisley, UK) for 10 min at 37°C and

then washed immediately with warm PBS containing 5 mM glucose. Fluorescence was measured without delay at an excitation wavelength of 485 nm and emission wavelength of 590 nm.

Intracellular total radical production

Total radical production was measured in intact leukocytes in 96-well plates following cell treatment as above. Briefly, leukocytes were washed with PBS before being loaded with 50 µM oxidation-sensitive 5-(6)-carboxy-2,7'-dichlorodihydrofluorescein diacetate (CDHFD) or 50 µM oxidation-insensitive 5,(6)-carboxy-2,7-dichlorofluorescein (CDF) for 60 min in the dark at 37°C. Cells were then washed twice with PBS, and the rate of total radical formation was determined by subtracting CDF fluorescence from CDHFD fluorescence by continuous recording until saturation was reached, at excitation/emission wavelengths of 485/530 nm [19].

Mitochondrial membrane potential

Mitochondrial membrane potential was analyzed in isolated leukocytes in 96-well plates using the fluorescent probe 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazol-carbo-cyanine iodide (JC-1). Briefly, following cell treatments, leukocytes were washed twice with PBS and then incubated for 30 min with 10 µg/ml JC-1 in PBS containing 5 mM glucose at 37°C in the dark. Following incubation, cells were washed twice with PBS, and the red and green fluorescence was measured immediately (excitation, 490 nm; emission, 590 or 520 nm). In intact mitochondria, 'J aggregates' form and JC-1 fluoresces red. When the mitochondrial membrane potential decreases, the JC-1 assumes a monomeric form and fluoresces green; results are expressed as the ratio of red to green fluorescence [20].

Oxygen consumption

Leukocyte oxygen consumption was measured after cell treatments using a Clark-type oxygen electrode. Briefly, 1 × 10⁶ cells were transferred to a 1-mL oxygen electrode chamber maintained at 37°C containing RPMI medium supplemented with 10% v/v FCS. Oxygen consumption was measured continuously for 15 min [21].

Phagocytosis

Neutrophils were isolated from whole blood using Polymorphprep™ (Axis-Shield, Oslo, Norway), diluted in RPMI 1640 containing 10% autologous serum, and used within 1 hr of isolation (viability >97%). *E. coli* strain 8959 (Public Health England, Porton Down, UK) were grown overnight in nutrient broth at 37°C with rotation at 250 rpm. Freshly prepared 2 × 10⁶ neutrophils were added to 2 × 10⁷ cfu *E. coli* plus 1 or 100 pg/mL melatonin or 6-hydroxymelatonin or solvent control. Identical control tubes were prepared without neutrophils to determine the starting number of bacteria and growth over the time course of the experiment. Tubes were rotated end-over-end at 37°C for 30, 60, and 90 min, and samples were

removed into ice-cold PBS and centrifuged at 4°C to pellet the neutrophils. The supernatant containing extracellular bacteria was removed, and the neutrophil pellet was resuspended in water at pH 11.0 and incubated at room temperature for 5 min and then vortexed thoroughly for 5 s to release intracellular bacteria. Each sample was diluted and spread on an agar plate and incubated overnight at 37°C, and colonies were then counted.

Statistics

Phase I dose escalation trial

Multilevel linear modeling was used to analyze oxygen saturation, blood pressure, heart rate, and ECG measures. Generalized linear models were used to analyze variables with two measures at baseline and 6 hr. The models all evaluated the effect of increasing melatonin doses on each outcome variable and were adjusted for age, weight, and height. Assumptions were checked, and where necessary, transformed data were used. The analyses were carried out using SPSS version 21 (IBM, Portsmouth, UK) or SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) using two-tailed tests with $P < 0.05$ used to indicate statistical significance. The visual analogue scores for the sleep scales were measured independently by two observers and entered into an Excel spreadsheet for summation of the total domain score. Data were analyzed nonparametrically. Each domain total from the three sleep scales was compared using the Friedman test for repeated measures, with Wilcoxon signed-rank post hoc test if $P < 0.05$. Domain totals between each dose cohort were compared using Kruskal–Wallis followed by Mann–Whitney U post hoc test if appropriate. Multiple comparisons were adjusted using Bonferroni correction where appropriate.

Ex vivo study

Data were not normally distributed and so are presented as median and interquartile range. Data were analyzed using Mann–Whitney U -test or Friedman's test, with either post hoc testing using Dunn's test for multiple comparisons or Page's L trend test as appropriate.

Results

Thirty-one subjects volunteered, but eight fulfilled the exclusion criteria and so were not recruited: Of these, five were taking regular medication or had chronic health conditions, two were occasional smokers, and one had a body weight >100 kg. Another three subjects consented but subsequently decided they could not spare the time. Twenty subjects aged 21–27 years completed the study visit and the 1-wk follow-up.

There was little difference in baseline characteristics between dose cohorts (Table 1). There were no grounds for noncontinuation, and the DMC recommended progression after each dose cohort. There were no serious adverse events and no adverse events attributable to the study drug. No subject reported nausea, headache, vomiting, diarrhea, or abdominal pain at any time during the

study visit or the 1-wk follow-up period. There were statistically significant decreases in some biochemical parameters between baseline and 6 hr, but none were of clinical significance and there were no effects of melatonin dose (Table 2). Analysis of physiological measurements over time revealed some statistically significant changes related to time (oxygen saturation, diastolic blood pressure) or dose (systolic blood pressure), but none of these changes were considered to be clinically significant or of any concern (Fig. 1).

Fifteen of the 20 subjects reported drowsiness at some point during the study visit. There were 17 separate time points at which subjects in the 20 mg dose cohort reported feeling sleepy, six in the 30 mg cohort, five in the 50 mg cohort, and 17 incidences in the 100 mg cohort; thus, there were as many reports of drowsiness for the 20 mg dose cohort as the 100 mg cohort and no obvious relationship to dose. All subjects were fully recovered by 6 hr and were able to go home alone.

Sleep disturbance is measured as mid-sleep awakening, wake after sleep onset, movement during sleep, soundness of sleep, and quality of disturbance, while latency characteristics include sleep latency and quality of latency. The maximum available total score for the sleep disturbance domain is 700, and a lower score indicates less sleep disturbance. The median [range] domain totals are given in Table 3. It can be seen that sleep disturbance was variable—but minimal—in most subjects and unrelated to the dose of melatonin. There was no difference in the time taken to fall asleep (sleep latency) or the sleep disturbance domain total between the three points at which sleep scales were completed.

Sleep efficiency is both the perceived quality and the duration of sleep. The maximum total score possible is 500 with a higher score representing greater sleep efficiency. Sleep efficiency was consistent and was similar in all subjects at all times, independent of melatonin dose (Table 3). There was no change in perceived sleep quality or the sleep efficiency domain total between any of the completed sleep scales in any of the dose cohorts.

The sleep supplementation domain measures how much extra sleep time subjects had during the day. The maximum total score possible is 400, and the higher the total score was, the more supplemental sleep was needed. We found that supplementary sleep duration was variable between individuals but again was unrelated to melatonin dose (Table 3). There was no difference in the total sleep periods between the sleep scales.

The technique for measuring melatonin was very sensitive with a lower limit of quantitation of 0.5 ng/mL. The

Table 1. Age, weight, and height data by dose cohort

Dose mg	Age years	Weight kg	Height cm
20	25 [23–26]	75 [73–94]	179 [173–203]
30	22 [21–22]	73 [57–99]	183 [170–191]
50	21 [21–27]	76 [73–86]	180 [178–191]
100	22 [21–22]	80 [73–85]	188 [179–193]

Median [range], $n = 5$ per dose cohort.

Table 2. Biochemistry/hematology data by dose cohort

Measure	Melatonin dose (mg)			
	20	30	50	100
Hemoglobin g/L				
Baseline	147 [139–161]	138 [127–142]	149 [142–169]	147 [145–148]
6 hr	145 [136–153]	133 [128–148]	145 [145–166]	141 [139–147]
Leukocytes ^a 10 ⁹ /L				
Baseline	5.0 [3.7–6.9]	5.6 [4.3–8.7]	4.9 [4.2–5.7]	5.3 [5.3–6.7]
6 hr	5.0 [4.2–6.7]	5.3 [4.2–6.6]	5.7 [4.3–6.7]	5.6 [4.2–6.4]
AST Units/L				
Baseline	33 [24–34]	25 [18–30]	24 [20–51]	23 [17–41]
6 hr	24 [17–25]	21 [16–29]	22 [17–33]	23 [16–36]
Bilirubin μ mol/L				
Baseline	17 [14–29]	16 [9–38]	18 [7–23]	11 [9–14]
6 hr	15 [12–29]	17 [8–35]	19 [9–23]	14 [9–17]
Creatinine μ mol/L				
Baseline	83 [74–86]	73 [70–96]	71 [68–77]	80 [62–85]
6 hr	73 [71–83]	68 [60–92]	66 [63–73]	79 [56–84]
Glucose ^b mmol/L				
Baseline	5.0 [4.3–5.3]	4.5 [3.7–5.6]	4.1 [3.6–4.7]	4.4 [3.9–5.0]
6 hr	4.4 [3.8–5.1]	4.4 [4.4–5.4]	4.0 [3.7–5.3]	4.2 [3.9–5.8]
Potassium ^c mmol/L				
Baseline	4.0 [3.9–4.6]	3.9 [3.5–4.2]	3.7 [3.6–3.8]	4.0 [3.6–4.0]
6 hr	3.8 [3.7–3.9]	3.9 [3.4–4.3]	3.8 [3.7–3.9]	3.8 [3.7–4.0]
Sodium ^d mmol/L				
Baseline	140 [140–141]	140 [138–140]	142 [140–143]	141 [140–143]
6 hr	141 [139–142]	140 [139–142]	141 [139–142]	141 [140–141]

Median [range], n = 5 per dose cohort.

^aBaseline >6 hr ($P < 0.001$) across all subjects, no effect of dose.

^bBaseline >6 hr ($P = 0.013$) across all subjects, no effect of dose.

^cBaseline >6 hr ($P < 0.001$) across all subjects, no effect of dose.

^dBaseline >6 hr ($P = 0.001$) across all subjects, no effect of dose.

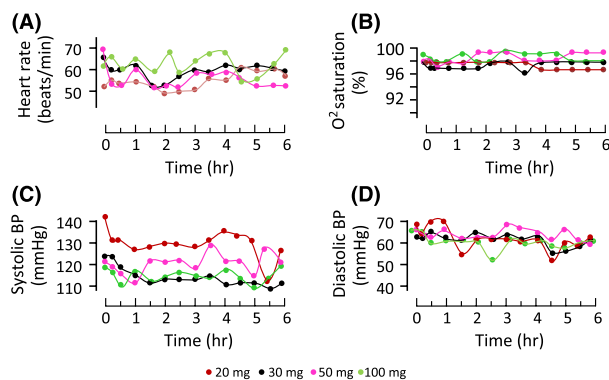


Fig. 1. Physiological measures in healthy subjects over 6 hr after receiving an oral dose of melatonin. (A) Heart rate, (B) oxygen saturation, (C) systolic blood pressure, and (D) diastolic blood pressure. Median values shown for clarity, n = 5 subjects per dose cohort. Multilevel linear modeling showed that oxygen saturation and diastolic blood pressure decreased over time, unrelated to dose ($P = 0.017$ and $P < 0.001$, respectively), and systolic blood pressure increased with melatonin dose ($P < 0.005$).

mean intra-assay precision (percentage relative standard deviation, n = 6) was 4.7% at 1 ng/mL and 2.6% at 80 ng/mL. Interassay precision was 4.1% at 1 ng/mL and 1.0% at 80 ng/mL, and recovery of melatonin added to serum was 96% at 0.5 ng/mL and 105% at 100 ng/mL. For 6-hydroxymelatonin sulfate determination in serum,

the mean intra-assay precision was 3.0% and the inter-assay precision was 5.0%. The precision of the urine 6-hydroxymelatonin sulfate assay was 4.1% intra-assay and 15.8% inter-assay.

In all subjects, melatonin was detectable in serum at 10 min after taking the oral dose and was rapidly cleared, with maximum levels reached at 30–60 min (Table 4). Melatonin concentration was variable between individuals even after the same melatonin dose, but there was a significant effect of melatonin dose on both the area under the concentration curve (AUC, $P = 0.0065$) and maximum concentration (C_{max} , $P = 0.0018$). Levels of 6-hydroxymelatonin sulfate were also detectable in serum by 10 min with maximum levels reached at a median of 120 min, but were less variable than melatonin, and remained broadly stable between 1 and 6 hr but were back at baseline levels at 24 hr (Fig. 2). Fig. 3 shows the relationship between melatonin and 6-hydroxymelatonin sulfate concentrations in individual subjects in the 50 mg dose cohort.

Urinary excretion of 6-hydroxymelatonin sulfate in the 6 hr after melatonin administration is shown in Table 5. There was a significant dose effect ($P = 0.0018$) although there was considerable inter-individual variation.

In the ex vivo study, IL-6 and IL-10 were both significantly increased in plasma from blood exposed to LPS/PepG (both $P < 0.0001$, Fig. 4) compared with solvent control. Exposure of blood to LPS/PepG plus melatonin or 6-hydroxymelatonin caused a significant dose-dependent

Table 3. Sleep scale domain total scores by dose cohort

Dose	Timing of sleep scale	Disturbance domain	Efficiency domain	Supplementation domain
20 mg	Before study visit	59 [16–423]	397 [310–461]	2 [2–109]
	Study visit	46 [8–145]	443 [324–490]	22 [0–94]
	Week after	99 [5–200]	429 [400–468]	2 [0–11]
30 mg	Before study visit	72 [36–180]	376 [313–420]	16 [7–79]
	Study visit	37 [29–142]	409 [272–487]	59 [4–139]
	Week after	81 [25–263]	313 [272–412]	36 [21–87]
50 mg	Before study visit	59 [11–104]	436 [268–456]	7 [0–160]
	Study visit	77 [13–234]	413 [312–458]	13 [2–35]
	Week after	60 [13–168]	399 [369–430]	1 [0–135]
100 mg	Before study visit	65 [61–464]	329 [317–453]	9 [0–154]
	Study visit	21 [10–205]	449 [251–458]	82 [49–137]
	Week after	95 [71–190]	379 [319–469]	18 [13–108]

Median [range], n = 5 per dose cohort.

No significant changes between time points or dose cohorts.

Table 4. Melatonin pharmacokinetic data in individual subjects

Dose and subject ID no.	AUC ^a ng/ml/min	Half-life mins	Cmax ^b ng/ml	Tmax min
20 mg				
1	1102	62.01	12.80	30.00
2	3266	52.62	35.70	60.00
3	3405	58.76	30.33	60.00
4	1564	50.38	20.86	30.00
5	13616	52.07	147.68	30.00
30 mg				
6	2491	48.33	30.09	30.00
7	1866	48.14	33.67	30.00
8	1304	71.62	12.95	30.00
9	822	63.22	6.25	60.00
10	1640	62.96	8.51	120.00
50 mg				
11	6034	55.58	75.42	30.00
12	1812	45.06	24.53	30.00
13	8915	51.39	113.78	30.00
14	4234	41.75	67.84	30.00
15	6554	53.09	91.38	30.00
100 mg				
16	9570	44.41	103.22	60.00
17	5639	29.47	94.00	60.00
18	18229	47.32	220.26	30.00
19	4458	45.18	47.71	60.00
20	11062	55.89	138.83	30.00

AUC, area under the concentration curve.

^aSignificant effect of dose ($P = 0.006$).

^bSignificant effect of dose ($P = 0.006$).

decrease in IL-6 (Fig. 4A), but IL-10 was unaffected by either drug (Fig. 4B). Likewise, LPO was significantly higher in blood exposed to LPS/PepG, and both melatonin and 6-hydroxymelatonin caused a significant dose-dependent decrease (Fig. 4C). The respiratory burst also increased upon exposure to LPS/PepG, and this was significantly decreased when blood was co-treated with LPS/PepG plus melatonin or 6-hydroxymelatonin (Fig. 5).

Exposure of isolated leukocytes to LPS/PepG resulted in a significant increase in mitochondrial membrane potential, total radical production, oxygen consumption, and mitochondrial superoxide production (Table 6). Co-exposure of cells to LPS/PepG plus melatonin or 6-hydroxyme-

Table 5. Urine 6-hydroxymelatonin in individual subjects

Dose and subject ID no.	Total 6-hydroxymelatonin excreted in 6 hr ^a mg	Median dose cohort total 6-hydroxymelatonin excreted in 6 hr mg
20 mg		
1	2.22	3.72
2	4.38	
3	3.72	
4	6.20	
5	2.96	
30 mg		
6	3.81	5.08
7	4.60	
8	8.80	
9	5.55	
10	5.08	
50 mg		
11	34.98	13.95
12	28.45	
13	13.95	
14	11.23	
15	5.82	
100 mg		
16	22.92	37.09
17	37.09	
18	47.09	
19	34.54	
20	66.03	

^aSignificant effect of dose ($P = 0.0018$).

latonin resulted in decreased membrane potential, radical production, and oxygen consumption (Table 6). When isolated neutrophils were cultured with *E. coli*, all bacteria were phagocytosed after 30 min regardless of treatment and there was no difference in the number of viable intracellular bacteria, showing that neither melatonin nor 6-hydroxymelatonin reduced neutrophil phagocytosis or killing of engulfed bacteria (data not shown).

Discussion

We found that large oral doses of melatonin in healthy volunteers were very well tolerated with no safety concerns and no clinically relevant changes in any physiological or

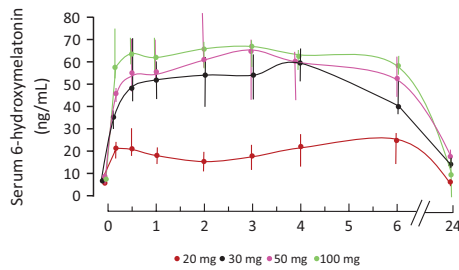


Fig. 2. Serum 6-hydroxymelatonin sulfate levels in healthy subjects over 6 hr after an oral dose of melatonin. Median and full range shown, n = 5 per dose cohort. There was a significant effect of dose ($P = 0.028$), and levels after 30, 50, or 100 mg were significantly higher than after 20 mg (all $P < 0.001$).

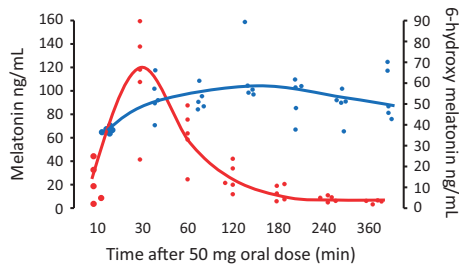


Fig. 3. Serum melatonin (red) and serum 6-hydroxymelatonin (blue) in individual subjects following an oral dose of 50 mg melatonin (n = 5 per dose cohort). Lines show median values.

biochemical measures. The concentrations of melatonin in the blood were variable and were rapidly cleared, but levels of the metabolite 6-hydroxymelatonin sulfate were more consistent and remained stable for several hours,

returning to baseline levels by 24 hr. The levels of both melatonin and 6-hydroxymelatonin achieved after oral dosing were shown to be bioactive in terms of antioxidant/anti-inflammatory effects in an ex vivo model of sepsis.

Sepsis can be defined as an uncontrolled immune and inflammatory response to an infectious insult, resulting in oxidative stress, massive cytokine release, and mitochondrial dysfunction. The role of damage to mitochondria in the pathophysiology of sepsis and subsequent organ dysfunction is widely accepted [3–5]. Melatonin is known to have a plethora of antioxidant and anti-inflammatory effects likely to be of benefit in sepsis [4, 5, 7–9].

Oral bioavailability of melatonin is low and has been estimated to be approximately 15% of the parent compound [22–24] with rapid clearance. The low bioavailability of melatonin and the marked interindividual variability that are reported here have been reported previously [24, 25] and are a consequence of variable first-pass extraction in the liver, due to genetic differences in the activity of cytochrome P450 enzymes, notably CYP1A1 but also CYP1A2, which convert melatonin to the 6-hydroxymelatonin metabolite before it enters the systemic circulation [26–28]. The majority of melatonin is converted to 6-hydroxymelatonin in the liver, then sulfated (~80%) or glucuronidated (~10%), and excreted in urine. The remainder can be demethylated back to *N*-acetylserotonin [29]. In addition, 6-hydroxymelatonin sulfate can be formed at extrahepatic sites by CYP1B1 [27]. In addition to enzymatic processes, 6-hydroxymelatonin can also be generated by nonenzymatic means via reaction with peroxynitrite or hydroxyl radical such that during oxidative stress, persistent production of 6-hydroxymelatonin might be expected.

Table 6. Mitochondrial function in isolated leukocytes

Melatonin or 6-hydroxymelatonin ng/ml	Mitochondrial membrane potential Red/green fluorescence ratio	Oxygen consumption nMoles O ₂ /min/mg protein	Total radical production ΔFluorescence x 10 ³ /min/10 ⁶ cells	Mitochondrial superoxide production Fluorescence units x 10 ³
Solvent control	23.9 [18.3–27.0]	17.9 [14.2–27.2]	56 [34–61]	260 [250–270]
Melatonin + LPS/PepG	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P = 0.0029$
0	28.6 [21.1–33.3] [#]	36.1 [23.0–56.2] [#]	88 [51–127] [#]	290 [270–310] [#]
0.01	21.6 [15.9–24.3] [*]	27.4 [18.6–38.6] [*]	59 [39–97] [*]	280 [270–300] [*]
0.1	19.9 [15.8–24.3] [*]	22.6 [16.3–32.4] [*]	62 [38–80] [*]	270 [250–300] [*]
1	21.0 [14.5–25.8] [*]	24.3 [18.2–41.5] [*]	62 [37–97] [*]	270 [260–300] [*]
10	19.6 [15.6–28.3] [*]	25.6 [20.5–36.8] [*]	60 [37–91] [*]	270 [250–300] [*]
100	23.2 [15.4–26.7] [*]	32.4 [25.7–43.4]	59 [35–111] [*]	270 [250–290] [*]
1000	24.5 [15.2–28.6] [*]	37.7 [28.1–48.4]	66 [43–105] [*]	270 [270–280] [*]
Solvent control	21.5 [16.1–23.3]	19.0 [14.9–30.9]	57 [41–70]	250 [230–280]
6-Hydroxymelatonin + LPS/PepG	$P < 0.0001$	$P = 0.0037$	$P < 0.0001$	$P = 0.0035$
0	29.2 [21.8–32.8] [#]	33.1 [21.1–53.4] [#]	89 [60–122] [#]	290 [260–300] [#]
0.01	21.1 [16.1–23.3] [*]	19.8 [13.8–26.7] [*]	76 [45–117] [*]	270 [250–300] [*]
0.1	20.4 [17.2–23.9] [*]	20.0 [12.9–25.2] [*]	61 [46–108] [*]	280 [270–300] [*]
1	21.5 [19.1–26.7] [*]	18.9 [15.1–25.2] [*]	68 [40–93] [*]	280 [260–300] [*]
10	18.9 [15.8–24.4] [*]	21.5 [14.8–25.9] [*]	64 [50–110] [*]	270 [250–300] [*]
100	22.2 [18.9–25.0] [*]	21.3 [15.3–26.7] [*]	70 [54–109] [*]	270 [260–300] [*]
1000	24.6 [16.6–27.8] [*]	19.4 [16.4–22.6] [*]	61 [47–72] [*]	270 [260–290] [*]

6-OH Melatonin = 6-hydroxymelatonin. Median [interquartile range], n = 20. *P*-value shown is Friedman's test across LPS/PepG groups. [#]Significantly different from solvent control (Wilcoxon signed ranks) and ^{*}significantly different from LPS/PepG alone (Dunn's test) $P < 0.05$.

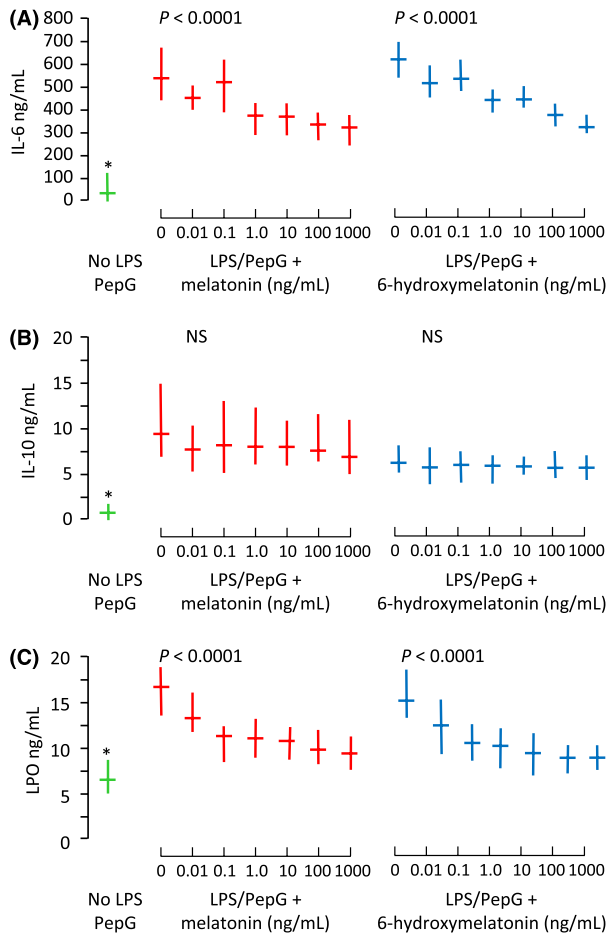


Fig. 4. (A) Plasma interleukin-6 (IL-6), (B) IL-10, and (C) lipid hydroperoxide (LPO) from whole blood treated with solvent control (green), lipopolysaccharide and peptidoglycan G (LPS/PepG) plus melatonin (red) or 6-hydroxymelatonin (blue). Median and interquartile range, $n = 20$ subjects. P -value is Page's trend test, and * = significantly lower than with LPS/PepG alone (Wilcoxon Signed Ranks, $P < 0.0001$)

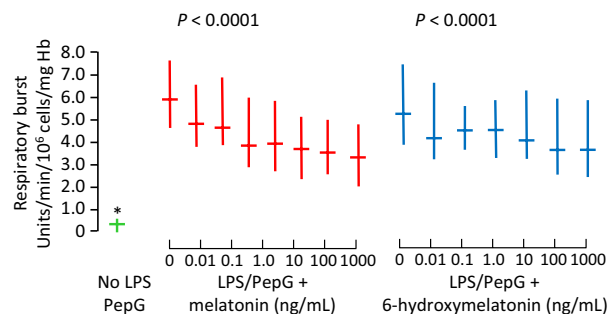


Fig. 5. Respiratory burst in whole blood treated with solvent control (green), lipopolysaccharide and peptidoglycan G (LPS/PepG) plus melatonin (red) or 6-hydroxymelatonin (blue). Median and interquartile range, $n = 20$ subjects. P -value is Page's trend test, and * = significantly lower than with LPS/PepG alone (Wilcoxon Signed Ranks, $P < 0.0001$)

We and others have reported a protective effect of melatonin in animal models of sepsis [7, 13–15, 30], suggesting a potential beneficial effect of melatonin in patients with

sepsis. It is now apparent that 6-hydroxymelatonin has similar antioxidant effects to melatonin and protects against various oxidative stress initiators [11, 29, 31–34] such that after oral dosing with melatonin, antioxidant effects may be mediated by 6-hydroxymelatonin. We undertook this dose escalation study to determine the tolerance of large oral doses of melatonin and the levels of melatonin and 6-hydroxymelatonin sulfate achieved and related this to a physiologically relevant ex vivo model of sepsis. Melatonin metabolism has been commonly monitored using measurement of urine excretion of 6-hydroxymelatonin sulfate, with maximum levels up to 2 mg/hr reported to occur between 04:00 and 08:00 but negligible amounts at other times [35]; however, there are few reports of 6-hydroxymelatonin in the serum after oral doses of melatonin in humans. Data from a single subject after an oral dose of 1 mg melatonin showed that plasma 6-hydroxymelatonin sulfate levels were elevated for 6 hr after melatonin administration, measured using radioimmunoassay, concurring with our findings [36]. In another study by Härtter and colleagues [37], a maximum plasma level of total 6-hydroxymelatonin from a single subject after taking 25 mg oral melatonin was reported to be approximately 600 ng/mL, again persisting for over 6 hr. Härtter et al. undertook enzymatic conversion of both the sulfate and glucuronidate forms of 6-hydroxymelatonin before liquid chromatography–mass spectrometry, to give the total conjugated 6-hydroxymelatonin level, but this technique incurred large losses during the hydrolysis step [37]. We found that 6-hydroxymelatonin was extremely unstable—hence our decision to measure 6-hydroxymelatonin in serum as the sulfated form using a validated enzyme immunoassay rather than using liquid chromatography. The long-term stability of 6-hydroxymelatonin sulfate has been previously reported [38].

In some countries such as the USA, melatonin is considered a food supplement, whereas in Europe, it is considered a neurohormone. The only licensed melatonin product in the UK is a slow-release melatonin agonist licensed for use in sleep disorders. In the present study, under EU legislation, melatonin was considered an investigational medicinal product and capsules were manufactured from chemically synthesized melatonin. Administration of melatonin in both preclinical and human studies, even at supraphysiological doses, has an excellent safety profile, although there is little in the way of robust toxicological investigation allowing an evaluation of risk in clinical trials [39]. We found that there was no evidence of side effects after single doses of up to 100 mg oral melatonin in healthy young men. Minor decreases in leukocyte count, glucose, potassium, and sodium were probably related to the dilutional effects of drinking water in subjects who had been fasting. There were also minor changes in oxygen saturation and blood pressure over time, but again, no values were of clinical significance or concern at any time point. Small decreases in systolic blood pressure and heart rate have been reported after a single dose of 5 mg melatonin at 14:00 or 21:00 hr in healthy subjects, but values remained well within normal ranges [40, 41]. There have been previous anecdotal reports of nausea, headache, and itching in

healthy subjects and various patient groups [42, 43]. However, there are few previous comprehensive reports of rigorous and comprehensive monitoring of physiological and biochemical parameters in different melatonin dose cohorts. Using a nonsuggestive approach, we had no reports of any symptoms at any dose, either immediately after dosing or up to 1 wk afterward. No effect of chronic administration of melatonin on a multitude of biochemical measures was also seen in previous randomized, double-blinded study of subjects taking 10 mg daily for a month [43], and there was also no difference in the incidence of adverse events such as nausea and headache between those taking melatonin and those given placebo [43].

Melatonin has been reported to cause drowsiness [44, 45] and indeed has been used at doses of 1–5 mg to treat sleep disorders and jet lag [46]. In our study, most subjects did report subjective drowsiness, and some, but not all, fell asleep, but the number of subjects who were drowsy or fell asleep appeared unrelated to the melatonin dose. All our subjects were lying supine on a bed in a warm and soporific environment; we therefore thought it likely that subjective drowsiness levels would be overestimated, and so we were somewhat surprised that more subjects did not report feeling drowsy after even the highest dose of melatonin. All subjects were alert and able to go home at the end of the study visit (6 hr after the melatonin dose). Melatonin also did not change the objective assessment of subsequent sleeping patterns in any subject. Although several subjects reported subjectively that they ‘slept very well’ the night of the study visit, this was not reflected in the recorded quality or duration of sleep in the completed sleep scales. The sleep scale used is a validated scale used extensively for assessing sleep efficacy and disturbance and the need for supplementary daytime sleep [16]. However, the day of the week on which the sleep scales were completed may of course have had an impact on the data, and any alcohol intake the day after the study visit or a week later may also have affected results.

It was reported previously that 9 mg oral melatonin at 09:30 reduced sleep latency (i.e., time to fall asleep), assessed using a multiple sleep latency test at 2-hr intervals, with subjects forced to stay awake in-between [47]. Another double-blind trial of up to 40 mg melatonin given at 10:00 showed decreased sleep latency, increased sleep duration, and decreased wake after sleep onset 2 hr after all doses of melatonin, measured polygraphically [44]. However, in a randomized double-blind study of 30 subjects given 10 mg melatonin daily at night for 28 day, polysomnographic recording revealed no effect of melatonin on sleep latency, total sleep time, amount of rapid eye movement (REM) sleep, sleep efficiency, or arousals [43]. The only significant finding in terms of sleep was shorter duration of stage 1 of non-REM sleep in the subjects taking melatonin compared to the placebo group, but no changes within the melatonin group before and after melatonin [43]. Similarly, in a double-blind study of 1 or 5 mg melatonin given at night, no effect on sleep latency or duration was found, recorded using EEG [48]. These reports suggest that any sleep-promoting effects of melatonin are short lived and effects seem to be more pronounced when melatonin is given during the day [49]. It is

also possible that subjects in some previous studies may have had an expectation of the possible effects of melatonin, which may have resulted in subjective reports of somnolence. Two small studies of low-dose oral melatonin administration in patients on the intensive care unit reported minor effects on sleep, but no effect on sedation requirements [50, 51].

We developed a whole blood model as a physiologically relevant representation of early events in sepsis. Modeling sepsis is not straightforward; animal models are fraught with controversy [52], and although possible, modeling sepsis in humans is unpleasant and at best can only actually model aspects of inflammation [53]. The interaction between immune responses associated with sepsis is complicated and difficult to replicate in cell culture systems, although whole blood models are potentially more physiologically relevant than single-cell-type models in terms of reflecting complex immune cell–cell interactions. We found that the respiratory burst of whole blood exposed to LPS/PepG was decreased by both melatonin and 6-hydroxymelatonin, as reported previously in isolated neutrophils from patients with pancreatitis [54]. Bacterial wall proteins such as LPS and PepG promote inflammation via engagement of toll-like receptors, leading to myD88-dependent inflammatory responses including the respiratory burst via NADPH oxidase activity [55, 56] and resulting in activation of signaling pathways culminating in cytokine release. Treatment of blood with melatonin or 6-hydroxymelatonin also resulted in dose-dependent suppression of LPS/PepG-induced increases in IL-6 and lipid hydroperoxide levels, while IL-10 was unaffected, confirming previous findings in models of sepsis [7, 14–16] and other disease models [57]. The mechanism of the effect of melatonin/6-hydroxymelatonin may be via antioxidant scavenging, but melatonin has also been reported to affect NADPH oxidase activity [58] and several transcription factor pathways involved in cytokine responses, possibly mediated through effects on mitogen-activated protein kinases (MAPK) [11, 57–59].

We also investigated the effect of melatonin and 6-hydroxymelatonin on aspects of mitochondrial function in isolated leukocytes exposed to LPS/PepG. Sepsis is associated with mitochondrial damage, which is thought to contribute to the pathophysiology of organ dysfunction [3–6]. In the present study, we exposed leukocytes to sepsis-like conditions for 18 hr to mimic the early stages of sepsis and found increased intracellular total radical production and intra-mitochondrial superoxide production, with concomitantly increased oxygen consumption, all of which were attenuated by melatonin and 6-hydroxymelatonin at all concentrations. We previously reported that melatonin and other related indoles, including 6-hydroxymelatonin, attenuated mitochondrial dysfunction induced by 7-day exposure to LPS/PepG in human endothelial cells [11]. Similar protective effects of melatonin on mitochondrial function have been reported in models of sepsis in animals [7, 30]. Mitochondrial membrane potential in leukocytes in the present study increased upon LPS/PepG exposure and was reduced to that seen in control cells by melatonin and 6-hydroxymelatonin. The increase in mitochondrial membrane potential is required physiologically to enhance the

bactericidal activity of phagocytes caused by the downregulation of mitochondrial uncoupling protein-2 [60]. The enhanced mitochondrial membrane potential is necessary to drive increased production of mitochondrial ROS (mROS) through boosting electron leak during oxidative phosphorylation. Evidence indicates that mROS is an important contributor to the bactericidal activity of the respiratory burst and innate immune signaling by augmenting pro-inflammatory cytokine production via nuclear factor κ B (NF κ B) and MAPK [61–64]. Treatment with melatonin/6-hydroxymelatonin resulted in a return of mitochondrial membrane potential to baseline, thus presumably contributing to the blunting of subsequent inflammatory responses. Of course, this may have had an unwanted effect of reducing phagocytic cell killing of bacteria. However, in additional studies we found that the ability of neutrophils to both phagocytose and kill ingested *E. coli* was unaffected by melatonin or 6-hydroxymelatonin at concentrations up to 100 pg/mL.

Melatonin has been given previously to patients with sepsis at relatively low doses, with the aim of influencing sleeping patterns [50, 51], but we suggest that higher doses of oral melatonin are likely to have beneficial effects on inflammatory responses and are probably primarily mediated via 6-hydroxymelatonin. A recently published study of 100 mg melatonin given as an intravenous infusion over an 8-hr period before injection of a very small dose of LPS in a human model of endotoxemia showed only minor effects of melatonin on cytokine levels and lipid peroxidation products [65]. However, the changes seen in response to LPS administration were small compared with those seen both in other human models of endotoxemia and in patients with sepsis [53]. Unfortunately, the levels of melatonin or its metabolites were not reported, nor the effects on sleep or other side effects, nor verification of the model in terms of inflammatory responses such as body temperature, hormone levels, and leukocyte counts [65].

In summary, we showed that the levels of both melatonin and 6-hydroxymelatonin achieved after oral dosing of melatonin were within the range of the doses at which antioxidant and anti-inflammatory effects were seen in blood cultured under conditions mimicking sepsis and that there were no side effects. The variable responses between individuals should be considered when melatonin is administered. We propose that administration of around 50 mg of melatonin would generate melatonin and—perhaps more importantly—6-hydroxymelatonin levels that correspond to concentrations at which attenuation of inflammatory and oxidative stress biomarkers was seen *ex vivo*, even in those subjects in whom melatonin levels were lowest.

Author contributions

HFG conceived of the study, contributed to study design, took overall trial management responsibility, recruited subjects and acquired data, and drafted and revised the manuscript. NRW contributed to the design and conduct of the study and subject recruitment and critically revised the manuscript. LSA contributed to study design, undertook statistical analyses, and critically revised the manuscript. DAL contributed to study

design, undertook sample preparation and laboratory analyses, and critically revised the manuscript. LA contributed to study design, conduct of the study, and data acquisition and critically reviewed the manuscript. GC developed and validated the melatonin assay and contributed to drafting of the manuscript. All authors read and approved the final article.

Acknowledgements

This study was funded by the Chief Scientist Office, NHS Scotland. We would like to thank all the volunteers who gave up their time and blood to take part in the study and the data monitoring committee and staff of the intensive care unit for their support. In addition, thanks to Dr Malachy Columb for performing Page's trend test for us and to Annette Fearnley at Nu-Pharm Ltd for her advice.

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