Melatonin Is Neuroprotective in Experimental Streptococcus pneumoniae Meningitis

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Neuronal injury in bacterial meningitis is a consequence of the direct toxicity of bacterial components and inflammatory and oxidative mechanisms. Adjunctive therapy with melatonin was investigated in vitro and in experimental meningitis. Cellular damage was reduced by treatment with melatonin in organotypic hippocampal cultures (P < .001) and in human SH-SY5Y cells (P < .01). Rabbits were infected intracisternally with Streptococcus pneumoniae and received either melatonin (20 mg/kg body weight/24 h; n = 12) or saline (n = 11) intravenously. Twelve hours later, all rabbits received ceftriaxone (10 mg/kg body weight/h). The density of apoptotic dentate granule cells was lower in melatonin-treated rabbits (81.8 ± 52.9 vs. 227.5 ± 127.9 cells/mm²; P = .002). The activity of superoxide dismutase in the hippocampal formation was higher (P = .003), and nitrite concentrations in cerebrospinal fluid were lower, after treatment with melatonin (P = .003). Melatonin reduced neuronal injury in vitro and in experimental meningitis, and it may be suitable as adjunctive therapy in human meningitis.

Neuronal injury in bacterial meningitis is a consequence of leukocyte invasion into the central nervous system (CNS), stimulation of microglia and resident macrophages, and direct toxicity of bacterial components on cerebral endothelium and neuronal cells [1]. The formation and release of free radicals is a key event in the cascade that eventually leads to neuronal injury. Pneumococcal cell-wall components attract leukocytes into the CNS that release reactive oxidants and proteolytic enzymes [2, 3]. Bacterial cell walls of Streptococcus pneumoniae and group B streptococci induce nitric oxide (NO) production in glial cells and induce neurotoxicity [4]. Moreover, the bacterium S. pneumoniae itself is able to release hydrogen peroxide [5, 6]. Treatment with the nonbacteriolytic antibiotic rifampin reduces the production of reactive oxygen species of cerebrospinal fluid (CSF) phagocytes and hippocampal injury in experimental S. pneumoniae meningitis [7]. Antiinflammatory and antioxidative treatment strategies are therefore promising with respect to minimizing cerebral complications in bacterial meningitis.

The antioxidant N-acetyl-5-methoxytryptamine (melatonin) is a derivate of the amino acid tryptophan. Physiologically, melatonin is the major secretory product of the pineal gland. It is also synthesized in extrapineal tissues, such as the retina and enterochromaffin cells of the gastrointestinal tract [8, 9]. Melatonin is released by the pineal gland in a diurnal rhythm and is involved in the regulation of the circadian rhythm, sleep, and reproduction [10]. So far, 2 classes of membrane-bound melatonin receptors, the G-protein coupled receptor family MT1 and MT2 and the quinone reductase enzyme family MT3, have been identified [11–14]. Moreover, melatonin may act by stimulating nuclear receptors [15, 16]. Both receptor-mediated and non–receptor-mediated effects have been proposed for melatonin. Melatonin is a potent broad-spectrum antioxidant. It scavenges a variety of oxygen and nitrogen species, including the hydroxyl radical, hydrogen peroxide, singlet oxygen, NO, and peroxynitrite, and it is irreversibly oxidized to 5-methoxy-N-acetyl-N-formyl-kynuramine.
[17]. Moreover, melatonin stimulates the expression of several antioxidative enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase [18–21].

High-dose administration of melatonin inhibits neuronal and glial injury in various models of disease, including cerebral artery occlusion and epileptic seizures [22–26]. Surgical removal of the pineal gland exaggerates cellular damage caused by free radicals during oxidative challenge [27, 28], which indicates that endogenous melatonin concentrations already possess antioxidative properties. Because oxidative mechanisms play a central role in the pathophysiology of bacterial meningitis and contribute to neuronal injury and brain damage [1–3], we studied the efficacy of melatonin in cell cultures exposed to S. pneumoniae and oxidative stress and in a rabbit model of pneumococcal meningitis.

MATERIALS AND METHODS

Organotypic hippocampal cultures. Six- to 8-day-old NMRI mice bred at the animal care facility of the Max-Planck-Institute for Biophysical Chemistry (Göttingen, Germany) were decapitated. The hippocampal formation was prepared and cut transversally with a McIlwain tissue chopper into 400-μm-thick slices under sterile conditions. Slices were kept in Grey’s balanced salt solution supplemented with 36 mmol/L d-glucose for 30 min at 4°C. Thereafter, slices were embedded in plasma clots on glass coverslips, which were then coagulated by the addition of thrombin. Coverslips were transferred to plastic culture tubes that contained culture medium composed of 50% Hanks’ basal medium, 25% Hanks’ balanced salt solution, 25% heat-inactivated horse serum supplemented with glutamine (1 mmol/L), and d-glucose (36 mmol/L). Culture tubes were placed in a roller device rotating at 10 revolutions/h in an air-ventilated incubator at 36°C. After 12 days, cultures were challenged with 10^7 cfu/mL of a living unencapsulated S. pneumoniae R6 strain for 48 h. The unencapsulated strain was chosen because the capsule masks the cell-wall epitopes that stimulate the innate immune system. Ceftriaxone (1 μg/mL) was administered at the same time, to prevent bacterial growth. Melatonin (Sigma-Aldrich) was dissolved in medium at a concentration of 0.1 μg/mL. Organotypic hippocampal cultures (n = 17 each group) were treated with S. pneumoniae R6 and ceftriaxone or with S. pneumoniae R6, ceftriaxone, and melatonin. Negative control cultures were not exposed to S. pneumoniae R6 and received only melatonin and ceftriaxone.

Propidium iodide (PI) staining of organotypic hippocampal cultures. The vital dye PI (Sigma-Aldrich) was used to determine cell-membrane damage in organotypic hippocampal cultures. With the loss of cell membrane integrity, PI enters the cell and binds to DNA. PI fluo escence is therefore related to necrotic or late apoptotic cell death. After treatment with S. pneumoniae and melatonin for 48 h, medium was replaced by PI (25 μg/mL dissolved in medium), followed by incubation for 1 h at 36°C. Thereafter, organotypic hippocampal cultures were examined by dark-field fluo escence microscopy (Zeiss; Axioshot). Fluorescence intensity was recorded by a CCD camera (Zeiss AxioCam). Subsequently, cultures were fixed with 4% buffered formaldehyde, to induce maximum membrane damage; washed briefly in 0.1 mol phosphate buffer; rehydrated with medium that contained PI; and again examined by dark-field fluo escence microscopy. Light intensity was quantified offline with the image analysis software Sigma Scan Pro (version 5.0; Jandel Scientifi Software). Cell damage induced in the dentate gyrus was calculated as the ratio of light intensity before fixatio divided by the light intensity after fixatio ×100 (percentage of PI uptake).

SH-SYSY neuroblastoma cell cultures. To examine the effects of melatonin on cell viability under conditions of oxidative stress, SH-SYSY human neuroblastoma cells were exposed to 3-morpholinosydnonimine (SIN-1; Calbiochem). At physiological pH, SIN-1 spontaneously decays to NO and superoxide anion radicals. SH-SYSY human neuroblastoma cells were maintained in RPMI 1640 medium (Biochrom) at 37°C with 5% CO₂. SH-SYSY cells were seeded into 96-well plates at a density of 10^4 cells/cm², and cultures were treated with medium that contained SIN-1 (500 μmol/L) or SIN-1 plus melatonin at concentrations of 0.1, 1, 10, 100, and 1000 μg/mL (n = 6 for each group). Four hours later, cell viability was determined.

Primary mouse microglial cell culture. Primary cultures of microglial cells were established from brains of newborn C57/BL6 mice (1–3 days old). After removal of the meninges, cells were mechanically dissociated and suspended in Dulbecco’s modifi Eagle medium with Glutamax I ( Gibco) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were plated at a density of 2 brains/T75 culture flask ( Corning Costar) and incubated at 37°C with 5% CO₂. After 10–14 days, the confluent mixed glial cultures were shaken 200 times/min for 30 min. Microglial cells in the supernatants were replated in 96-well cell-culture plates at a density of 75,000 cells/well. Stimulation of microglial cells was performed with the Toll-like receptor (TLR)-2 agonist tripalmitoyl-s-glycerol-cysteine (Pam3Cys-OH; EMC-Microcollections) for 24 h in the presence of interferon (IFN)–γ (100 U/mL). Cultures were treated with Pam3Cys (0.1 μg/mL) or Pam3Cys plus melatonin at concentrations of 10, 100, 300, and 1000 μg/mL (n = 8 for each group). Negative control cultures received IFN-γ only. The release of NO into the supernatant was quantified by measurement of the stable reaction product nitrite, by use of the Griess reagent. Microglial cells were assayed for cell viability.

Measurement of cell viability. The cell viability of SH-SYSY and microglial cells was determined by use of the WST-1 cell proliferation reagent (Roche Applied Science). The assay
is based on the cleavage of the tetrazolium salt WST-1 by active mitochondria, which produces a soluble formazan. Cells were incubated with WST-1 for 2 h. Then, the formazan dye formed was quantified by measuring the optical density at 490 nm by use of a Genios multiplate reader (Tecan). The absorbance directly correlates with the number of metabolically active cells.

**Rabbit model of experimental meningitis.** A penicillin-sensitive *S. pneumoniae* type 3 strain originally isolated from an adult patient with meningitis (MIC, 0.03 µg/mL; minimal bactericidal concentration, 0.06 µg/mL) was used (gift from M. G. Täuber, University of Bern, Switzerland). After the intramuscular administration of anesthesia with ketamine (25 mg/kg body weight) and xylazine (5 mg/kg body weight), New Zealand White rabbits were inoculated intracisternally by suboccipital puncture with 1 × 10⁶ cfu of *S. pneumoniae*. Thereafter, rabbits received, intravenously, either 20 mg/kg body weight melatonin (Sigma-Aldrich) dissolved in 50 mL of saline (n = 12) or an equal amount of saline (n = 11), by a continuous infusion, for 24 h. Anesthesia was maintained by the intravenous administration of urethane for the entire duration of the experiment (24 h). Ceftriaxone (Rocephin; Hoffmann-LaRoche) was administered intravenously for 12–24 h after infection was quantified by the measurement of nitrite, by use of Griess reagent; 100 µL of the samples was mixed with 100 µL of Griess reagent (equal volumes of 1% sulfonamide in 30% acetate and 0.1% N-[1-naphthyl]ethylenediamine in 60% acetate) in a 96-well plate. After 10 min, the optical density at 570 nm was measured with a Genios multiplate reader (Tecan). Concentrations were calculated by comparison of absorptions with a standard curve.

**RIA for the quantification of melatonin.** Melatonin concentrations in serum and CSF were measured by RIA that used a specific antibody (G/S 704-6483; Guildhay Antisera). The detection limit of the assay (at which 5% of the ligand is displaced) was 1 pg/300 µL. Intrastriatal and interassay variation were 6% and 12%, respectively. The results obtained with the RIA technique were validated by high-performance liquid chromatography measurements with electrochemical detection [29, 30].

**Statistics.** Data were expressed as means ± SDs. Groups from in vitro experiments were compared by the 2-tailed parametric 1-way analysis of variance, and P values were adjusted for repeated testing by use of Bonferroni’s multiple comparison test. Data from animal experiments were compared by unpaired t test. Bacterial titers in CSF were used for log-linear regression analysis. P < .05 was considered to be statistically significant.

**RESULTS**

**Protection by melatonin against cellular damage in *S. pneumoniae*-treated organotypic hippocampal cultures and in SIN-1–treated human SH-SY5Y cells.** In organotypic hippocampal cultures exposed to a pneumococcal R6 strain, cellular damage...
in the dentate gyrus, as indicated by PI fluorescence, was lower after treatment with melatonin at a concentration of 0.1 μg/mL ($P < .001$). Negative control cultures without exposure to S. pneumoniae showed either very low or no fluorescence (figure 1).

In SH-SY5Y cells, melatonin was capable of reducing oxidative cell damage caused by treatment with SIN-1 ($P < .01$). The concentrations required for neuroprotection, however, were higher than those that were effective in organotypic hippocampal cultures. The observed effect was dose dependent: melatonin at a concentration of 1 μg/mL substantially reduced cellular damage. Protection was maximal at 10 μg/mL. Higher concentrations of melatonin did not increase the protective effect. Neuroprotection was abolished at a concentration of 1 mg/mL (figure 2A).

**Lower nitrite concentrations in microglial cell cultures treated with melatonin.** Nitrite concentrations in supernatants of microglial cell cultures after stimulation with the TLR2 agonist Pam3Cys were reduced by treatment with melatonin ($P < .001$).

This effect was dose dependent, reaching a maximum at a concentration of 1 mg/mL (figure 2B). The decrease in nitrite concentrations in supernatants was not caused by toxic effects of melatonin: cell viability, as determined by the WST-1 cell proliferation reagent, showed no significant differences between melatonin-treated and control cultures (data not shown).
Table 1. Parameters of meningeal inflammation in cerebrospinal fluid (CSF) and concentrations of melatonin in CSF and serum of rabbits 12 and 24 h after the induction of bacterial meningitis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ceftriaxone treatment, h</th>
<th>Melatonin and ceftriaxone treatment, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein level, mean ± SD, mg/L</td>
<td>1590 ± 1498</td>
<td>1304 ± 902</td>
</tr>
<tr>
<td>Lactate concentration, mean ± SD, mmo/L</td>
<td>4.0 ± 2.1</td>
<td>3.4 ± 1.0</td>
</tr>
<tr>
<td>White blood cell count, mean ± SD, µL</td>
<td>846 ± 1803</td>
<td>441 ± 807</td>
</tr>
<tr>
<td>Melatonin concentration in CSF, mean ± SD, ng/mL</td>
<td>0.2 ± 0.2</td>
<td>80.1 ± 41.8</td>
</tr>
<tr>
<td>Melatonin concentration in serum, mean ± SD, ng/mL</td>
<td>0.5 ± 0.4</td>
<td>941 ± 727a</td>
</tr>
</tbody>
</table>

* P < .001 vs. control.

**CSF parameters and concentrations of melatonin in experimental pneumococcal meningitis.** Bacterial titers (mean ± SD) in CSF determined 12 h after inoculation were 6.3 ± 0.4 log cfu/mL in melatonin-treated rabbits and 6.5 ± 0.9 log cfu/mL in control rabbits (P = .5). The bactericidal rate was −0.63 ± 0.14 Δlog cfu/mL/h in rabbits that received adjunctive melatonin therapy and −0.63 ± 0.08 Δlog cfu/mL/h in control rabbits treated with ceftriaxone alone (P = .95). CSF lactate levels, protein concentrations, and WBC counts increased during the course of the experiment in both groups, and no significant differences between melatonin-treated and control rabbits were noted (table 1). Twenty-four hours after infection, the CSF concentration of nitrite, a stable reaction product of the reactive oxygen species NO, was lower in rabbits treated with melatonin and ceftriaxone, compared with rabbits treated with ceftriaxone alone (1.66 ± 0.15 vs. 2.41 ± 0.76 µmol/L; P = .003).

Melatonin readily entered the CSF. Levels in CSF 24 h after the start of treatment were ~20% of the corresponding serum concentrations. In melatonin-treated rabbits, CSF concentrations increased from 0.03 ± 0.02 ng/mL before treatment to 124 ± 86.1 ng/mL 24 h later (table 1).

**Marked decrease of neuronal cell death in experimental pneumococcal meningitis after treatment with melatonin.** The frequency of apoptotic neurons in the dentate gyrus of the hippocampal formation was lower after treatment with melatonin and ceftriaxone than after treatment with ceftriaxone alone (81.8 ± 52.9 vs. 227.2 ± 127.9 cells/mm²; P = .002) (figures 3A and 4).

Figure 3. Density of apoptotic neurons in the dentate gyrus of the hippocampal formation (A) and activity of superoxide dismutase (U/10 mg tissue) in the hippocampal formation (B) 24 h after infection in rabbits treated with ceftriaxone and melatonin (melatonin) or ceftriaxone alone (control) (mean ± SD; **P = .002 and *P = .04, melatonin vs. control).
Increased activity of SOD in melatonin-treated rabbits. In brain homogenates of the hippocampal formation, the activity of SOD was higher in rabbits treated with melatonin than in control rabbits. SOD concentrations were 6.29 ± 2.24 U/10 mg of hippocampal tissue after treatment with melatonin and 4.29 ± 1.65 U/10 mg in control rabbits that received only ceftriaxone (P = .04) (fig. e 3B).

DISCUSSION

The formation of reactive oxygen species and the failure of endogenous antioxidant mechanisms has been considered to be important for cellular damage in aging and in a variety of neurodegenerative diseases [31–34]. Similarly, oxidative mechanisms contribute to acute brain damage, such as cerebral ischemia [34]. The excessive formation of reactive oxygen and nitrogen species has also been considered to be a key event in the pathophysiology of bacterial meningitis. Hydroxyl radicals and other compounds mediate cell death by membrane peroxidation, breakdown of the protein structure, and DNA damage. Finally, intracellular calcium increase, energy depletion, and caspase activation are the effectors of cell death in meningitis [1, 3]. Several antioxidant treatment strategies have been tested so far in bacterial meningitis. The radical scavenger α-phenyl-tert-butyl nitrite (PBN) prevented hippocampal and neocortical injury in an infant rat model of group B streptococcal meningitis [2]. The antioxidants N-acetylcysteine, deferoxamine, and trylizad-mesylate reduced cortical injury, but not hippocampal cell death, in S. pneumoniae meningitis [35]. Conversely, hippocampal damage and learning deficit were more pronounced after treatment with PBN in the same model [36]. Whether neuroprotection or detrimental side effects prevail depends not only on the animal model and the causative organism used but also on the toxic side effects of the antioxidant drug [36, 37].

The antioxidant melatonin is tolerated in large doses by humans and animals without producing severe adverse effects. In rats, melatonin administered at concentrations of 200 mg/kg body weight/day had no maternal toxicity and no detrimental effects on prenatal survival, fetal body weight, or the incidence of fetal malformations [38]. Melatonin is a compound that is endogenously produced at concentrations that are effective to protect against the oxidative stress that accompanies aging or neurodegenerative disorders [28, 32]. In acute cerebral diseases, however, adjunctive treatment with melatonin maximizes antioxidative effects. This strategy has been investigated in various experimental models of CNS injury but not yet in bacterial meningitis. In models of head trauma and cerebral ischemia, treatment with melatonin reduced the volume of contusion or cerebral infarction [25, 26, 39, 40].

In various models of bacterial meningitis and in human autopsy cases, neuronal injury has been frequently shown to occur in the hippocampal formation, and the apoptosis of dentate granule cells is a common feature [36, 41–43]. In the present study, organotypic hippocampal cultures exposed to S. pneumoniae were used as a model of brain-tissue damage in bacterial meningitis. Both apoptotic and necrotic cell death has been observed in this model [44]. Melatonin was capable of reducing cellular damage in the dentate gyrus, as indicated by PI fluorescence. Similarly, in human SH-SY5Y cells treated with SIN-1 to induce oxidative stress, melatonin alleviated cellular damage. In the rabbit model of ceftriaxone-treated pneumococcal meningitis, neuronal damage in the hippocampal formation was significantly reduced by melatonin administered continuously during the experimental period.

The nitrite concentration both in the supernatant of Pam3Cys-stimulated microglial cells and in the CSF of infected rabbits was reduced by melatonin. The effective melatonin concentrations, however, were higher in cultures of single cell types.
than in organotypic hippocampal cultures and in vivo. Melatonin has been shown to increase mRNA expression and the activity of glutathione peroxidase and SOD [18, 19]. In accordance with these findings, the activity of SOD was increased in the hippocampal formation of melatonin-treated rabbits in the present study. The neuroprotective effects of melatonin in complex systems are probably mediated not only by direct scavenging of free radicals but also by the stimulation of SOD and, possibly, other antioxidant enzymes.

Immunomodulatory effects of melatonin have been postulated [45]. Results of studies, however, concerning melatonin as a regulator of the immune system have been inconsistent. Cytokine production in lipopolysaccharide-stimulated macrophage and microglial cell lines was not altered by melatonin, which suggests that melatonin is not a prominent modulator of macrophage and microglia function [46]. Consistent with this notion, melatonin did not influence parameters of inflammation within the subarachnoid space (CSF lactate levels, protein concentrations, and number of leukocytes) in the present study.

Melatonin also influences the expression of neurotrophic factors, which may promote cell survival: in rat glioma cells and in dopaminergic striatal neurons, melatonin induced an enhanced mRNA expression of glial cell line–derived neurotrophic factor [47, 48]. In contrast to other antioxidants—such as N-acetylcycteine or deferoxamine, which, in experimental meningitis, relieved only neocortical neuronal injury and not hippocampal damage [35]—melatonin was effective in reducing neuronal apoptosis in dentate granule cells, the most frequent site of neuronal injury in bacterial meningitis [42]. This may be of particular importance, because dexamethasone—by decreasing mortality and the risk of severe neurological sequelae in adults with bacterial meningitis [49]—aggravated hippocampal injury and spatial learning deficit in animals [41, 50]. Studies have investigated the long-term effects of melatonin or of a combination of melatonin and dexamethasone on neuropsychological function are necessary.

Pharmacokinetic data qualify melatonin for adjunctive therapy in acute cerebral diseases: melatonin readily penetrates the blood–CSF barrier and cell membranes. The CSF:plasma concentration ratio has been reported to be ∼0.38 [51]. In our experiments, CSF levels of melatonin 24 h after the start of treatment were ∼20% of the corresponding serum concentrations. Melatonin concentrations of 0.1 μg/mL, which reduced cellular damage in organotypic hippocampal cultures, were achieved in the CSF of rabbits treated with the 20 mg/kg body weight dose (table 1).

In conclusion, melatonin was protective in organotypic hippocampal cultures treated with S. pneumoniae and in human SH-SY5Y cells exposed to oxidative stress. In experimental pneumococcal meningitis, melatonin reduced neuronal injury by direct and indirect antioxidative mechanisms. Low toxicity and the ability to readily penetrate the blood–CSF barrier qualify melatonin as a candidate for adjunctive therapy in bacterial meningitis.

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References


