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Melatonin Limits Glial Calcium Influx, Reduces Pro-inflammatory Cytokine Level, and Rescues Hippocampal Neurons of Adult Rats with Acute *Klebsiella pneumoniae* Meningitis

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ABSTRACT

Acute bacterial meningitis caused by *Klebsiella pneumoniae* (KP) is the major health threat with high mortality rate and severe neuro-cognitive sequelae. The intense pro-inflammatory cytokine released from calcium-mediated microglia activation plays an important role in eliciting extensive neuronal damage in hippocampal regions. Considering melatonin possesses significant anti-inflammatory and immuno-modulatory properties, the present study is aimed to determine whether melatonin would effectively decrease the inflammatory responses and prevent hippocampal damage in animals subjected to KP. Adult rats inoculated with KP were received melatonin injection immediately at doses of 5, 25, 50 and 100 mg/kg. Following 24 h of survival, all experimental animals were processed for time-of-flight secondary ion mass spectrometry (TOF-SIMS) (for detecting glial calcium intensity), isolectin-B4 (GSA-IB4) histochemistry (reliable marker for microglia activation), pro-inflammatory cytokine assay as well as cytochrome oxidase (COX) and *in situ* dUTP end-labeling (TUNEL) (representing neuronal bio-energetic status and apoptotic changes, respectively). Results indicated that in KP-infected rats, numerous calcium-enriched microglia, enhanced pro-inflammatory cytokine and various apoptotic neurons with low bio-energetic activity were detected in hippocampal regions. However, following melatonin administration, all parameters including glial calcium intensity, microglia activation, pro-inflammatory cytokine level as well as apoptotic neurons were successfully decreased with maximal change observed in dose of 100 mg/kg. Enzymatic data corresponded well with above findings in which all survival neurons displayed high bio-energetic activity. As effectively reducing glia-mediated inflammatory response is neuro-protective to hippocampal neurons, the present study thus supports the clinical use of melatonin as a potential therapeutic strategy to counteract KP meningitis-induced neuro-cognitive damage.

INTRODUCTION

Acute *Klebsiella pneumoniae* (KP) meningitis has recently become an increasingly common cause of central nervous system infection via both community acquired and nosocomial route of transmission in developing countries [1,2]. Although the early diagnosis and antimicrobial therapy are becoming much advanced in current society, KP meningitis is still associated with a high mortality rate and incidence of neuro-cognitive sequelae [3,4]. Previous studies have indicated that over-reactive inflammatory responses induced by invading pathogen may play an important role in eliciting lots of functional and structural brain damages [5,6]. Pathological reports also demonstrated that enhanced pro-inflammatory cytokine release [e.g. tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6)] triggered by microglia activation may serve as the underlying mechanisms for neuronal apoptosis in hippocampal regions [7-9]. It has recently been reported that the extent of microglial activation is largely dependent on its intracellular calcium (Ca^{2+}) levels [10]. The Ca^{2+}-mediated microglia activation would regulate a lot of microglial functions including proliferation, migration, ramification / deramification, and pro-inflammatory cytokine release after bacterial exposure or immunological stimuli [10,11]. As exaggerated pro-inflammatory cytokine release would contribute to the breakdown of blood brain barrier (BBB) and interrupt the bio-energetic activity or metabolism of injured neurons [12], exogenous treatment of anti-inflammatory or immuno-modulatory agents that can successfully suppress the microglia activation and reduce the inflammation processes would therefore be of great potential for clinical use as therapeutic strategy to counteract the KP meningitis-induced neuro-pathological damage [13].

Melatonin, the chief secretory product of pineal gland, is best known for its effects on circadian rhythmicity, reproductive function, free radical scavenging and anti-oxidative activity [14-16]. Within the past few years, the anti-inflammatory and immuno-modulatory properties of

melatonin in improving the functional recovery of injured neurons have been well documented [17-22]. It has been reported that melatonin can reduce the production of several pro-inflammatory cytokines and decrease the severity of neuronal damage resulted from a variety of experimental neuro-pathologies [23-27]. Pharmacological studies also demonstrated that melatonin can prevent hippocampal degeneration and improve the cognitive function through microglial inhibition after direct exposure to neuro-toxic agents [28,29]. It is indicated that melatonin can significantly modulate the intracellular calcium signaling and alter the immune potentiality of glial cells [30,31]. By successfully repressing the genetic expression of glial related cytotoxic factors (e.g. nitric oxide or superoxide anions) and pro-inflammatory cytokines, melatonin could inhibit microglia activation and therefore reduce the glia-mediated inflammatory responses [32-34]. With regard to the viewpoint that intense microglia activation may also serve as the underlying mechanism participated in the formation of hippocampal apoptosis and cognitive deficiency following bacteria infection [7-9], extensively examine the potential advantage of melatonin in reducing the neuronal damage induced by KP will not only help us to better understand the neuro-protective effects of melatonin but also provide important insights for clinical use of melatonin as a powerful therapeutic strategy to counteract the socio-economic costs arisen from KP meningitis in developing countries [35].

However, as far as we known, although the detrimental role of microglia activation and subsequently pro-inflammatory cytokine release in developing the hippocampal neuropathy following bacterial infection has been well documented, the detail expression of hippocampal pro-inflammatory cytokine as well as spatial distribution of activated microglia after KP meningitis has not been reported. Moreover, whether melatonin would exert its neuro-protective effects by suppressing KP-induced inflammatory responses and prevent neuronal apoptosis is still remained to be explored. Seeing that increased intracellular calcium level is a prerequisite for

microglia activation [10], the present study is aimed to determine whether melatonin would successfully limit glial calcium influx and consequently decreasing the pro-inflammatory cytokine level by the use of time-of-flight secondary ion mass spectrometry (TOF-SIMS, an instrument capable of quantifying the *in vivo* calcium intensity) [36], isolectin-B4 histochemistry (GSA-IB4, a reliable marker for microglia activation) [37] together with enzyme-linked immunosorbent assay (ELISA, a specific method suitable to evaluate the pro-inflammatory cytokine expressions) [38]. In addition, in order to examine the correlation between hippocampal apoptosis and pro-inflammatory cytokine release induced by KP infection, the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was also performed to clarify the interaction and subsequently expose the anti-apoptotic functions of melatonin. Furthermore, as hippocampal apoptosis would contribute to the disruption of bio-energetic status of injured neurons, the cytochrome oxidase (COX) histochemistry was further processed to assess the metabolic activity of hippocampal neurons [39] subjecting to acute KP meningitis in the current study.

MATERIALS AND METHODS

Treatment of experimental animals

Adult male Wistar rats (n = 60, weighing 200 ~ 250 g) obtained from the Laboratory Animal Center of the National Taiwan University were used in this study. All experimental animals were firstly divided equally into two groups. Animals in the first group were subjected to intra-ventricular *K. pneumoniae* injection while others in the second group were subjected to sham-operation without any bacterial exposure. After anesthetized with 7 % chloral hydrate (0.4 mL/100g), all animals were positioned in a stereotaxtic frame with a midline sagittal incision made in the head. Burr holes drilled in the skull over the lateral ventricles were used according to the parameters described previously (i.e. 0.8 mm posterior to bregma, 1.5 mm lateral to sagittal suture, and 3.6 mm beneath the surface of the brain) [40]. *K. pneumoniae* (Courtesy of Prof. Y-C Lai, Department of Microbiology and Immunology, Chung Shan Medical University) with the concentration of 1×10^6 colony forming units (CFU) in a volume of 10 μL was then intracerebroventricular inoculated in the ventricular space. After the operation, both experimental groups were further divided into five subgroups with 6 in each. The first subgroup remained un-treated, while subgroup II to V were received Ringer's solution or melatonin administration at doses of 5, 25, 50 and 100 mg/kg, respectively, 1 h following the operation. Melatonin (Sigma, St. Louis, MO, USA) was first dissolved in absolute alcohol and then diluted in Ringer's solution with final ethanol concentration less than 1%. All experimental animals were housed under same conditions with controlled temperature and exposed to an automatically regulated light:dark cycle of 12:12. In the care and handling of all experimental animals, the Guide for the Care and Use of Laboratory Animals (1985) as stated in the United States NIH Guidelines (NIH publication no. 86-23) were followed. All experimental procedures with bacterial exposure and melatonin administration were also approved by the Laboratory Animal Center Authorities of the Chung

Shan Medical University.

Collection of cerebrospinal fluid

The cerebrospinal fluid (CSF) was collected in all experimental animals with or without melatonin treatment 24 h after the KP inoculation. A 23G needle of winged infusion set was inserted through the midline of the atlanto-occipital membrane and aspirated gently with a 1 mL syringe connected to a silane tubing of the winged set. The leukocytes of the CSF were then counted in a Bűrker chamber in which samples with > 50 erythrocytes/μL were discarded.

Perfusion and tissue preparation

Following 24 h of bacterial inoculation, half of the experimental animals (n = 30) from all subgroups were deeply anesthetized with 7% chloral hydrate (0.4 mL/100g) and subjected to transcardiac perfusion with 100 mL of Ringer's solution, followed by 45 min of fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. During perfusion, the blood samples were drew out immediately from the left ventricle by cardiac puncture with a syringe and collected in a microtainer (Becton Dickinson, Sparks, MD, USA). After perfusion, the forebrain containing the hippocampus was removed and kept in a similar fixative for 2 h. The tissue block was then immersed in graded concentrations of sucrose buffer (10-30%) for cryoprotection at 4℃ overnight. Serial 20-μm-thick sections of the hippocampus were cut transversely with a cryostat (CM3050S, Leica Microsystems, Wetzlar, Germany) on the following day and were alternatively placed into five wells of a cell culture plate, such that each well ultimately contained a group of sections spaced 100 μm apart. Sections collected in the first well were processed for TOF-SIMS analysis, and those in the second well for TUNEL staining. The sections placed in the third well were processed for cytochrome oxidase histochemistry, and those in the fourth well for GSA-IB4 lectin labeling. In order to quantify the neuronal numbers, the regular hematoxylin-eosin (HE) staining was carried out in the last well.

Serum pro-inflammatory cytokine assay

The blood sample collected during perfusion was firstly centrifuged at 12,000 rpm for 5 min to separate the serum at room temperature. Following that, the serum pro-inflammatory cytokine level was measured by using the multiplexed bead-based cytokine immunoassays (Bio-Rad, Hercules, CA, USA) combined with a Cytokine Reagent Kit (#171-304000; Bio-Rad) and a Bio-Plex^{TM} Diluent Kit (#171-305008; Bio-Rad) [9,38]. The assay allows the quantification of several immuno-mediators in a sample volume as little as 12 μL.

TOF-SIMS analysis for calcium intensity and molecular image

The *in vivo* calcium intensity and the molecular image of calcium were assessed by the use of TOF-SIMS analysis. TOF-SIMS analysis was carried out on a TOF-SIMS IV instrument (ION-TOF GmbH, Münster, Germany) as described in our previous studies [41,42]. The gallium (Ga^+) ion gun operated at 25 kV was used as the primary ion source (1 pA pulse current) for experiments conducted in the current study. The Ga^+ primary ion beam was scanned over an area of 100 μm^2. Positive secondary ions flying through a reflectron mass spectrometer were detected with a micro-channel plate assembly operating at 10 kV post-acceleration. Mass calibration of the ion spectrum was achieved by using a set of mass peaks like *m/z* 15 (CH_3^+), 41 ($C_3H_5^+$), 69 (Ga^+), and paraformaldehyde molecule since this element was the major component in the tissue matrix following vascular fixation [41]. The ions related to Ca^{2+} (*m/z* 40.08) were used to identify and evaluate the molecular image of calcium expression.

TUNEL staining

To detect the potential DNA fragmentation in cell nuclei arose from apoptotic changes, the TUNEL reaction was applied to the sections collected in the second well. After several washed in 0.1 M PB, the sections were treated with 20 g/mL proteinase K for 10 min, followed by 0.3 % H_2O_2 in methanol for 10 min and 0.1 % Triton X-100 in 0.1 % sodium citrate for 2 min on ice.

After that, the sections were incubated with TUNEL reaction mixture (Roche, Berlin, Germany) for 60 min at 37℃. The reaction product was stained with diaminobenzidine (DAB) solution for 10 min at room temperature.

Cytochrome oxidase histochemistry

The slightly modified method of Wong-Riley [39] was used to demonstrate the COX reactivity in our present study. The reaction medium contained 0.03 % cytochrome c, 0.05 % 3,3'-diaminobenzidine and 0.02 % catalase (all from Sigma, St. Louis, MO, USA) in 0.1 M PB, pH 7.4. The sections collected from the third well were incubated with this medium at 4℃ in the dark overnight. After incubation, sections were rinsed for 20 min in 0.1 M PB followed by a rinse in distilled water to terminate the reaction.

GSA-IB4 histochemistry

For the detection of microglial cells in hippocampus after bacterial inoculation, sections collected in the fourth well were pre-incubated in 0.05 % H_2O_2 for half an hour to exclude the staining of endogenous peroxidase in lysosomes. Following a brief wash with 0.05 M Tris buffer (pH 7.4), sections was incubated in a lectin solution containing 0.025 mg/mL GSA-IB4 conjugated with horseradish peroxidase (HRP) (Sigma, St. Louis, MO, USA), 0.1 % Triton X-100, and 0.05 M Tris buffer saline overnight at 4℃. The labeling reactions were visualized with the diaminobenzidine.

Measurement of hippocampal pro-inflammatory cytokines

The concentration of hippocampal pro-inflammatory cytokines was determined by using the enzyme-linked immunosorbent assay (ELISA). After 24 h of bacterial inoculation, another half of the experimental animals (n = 30) from all subgroups were deeply anesthetized and decapitated. Following that, the forebrain segment containing the hippocampus was rapidly removed and homogenized with a glass homogenizer in 1 mL buffer containing 1 mmol/L

phenylmethylsulfonyl fluoride (PMSF), 1 mg/L pepstatin A, 1 mg/L aprotinin and 1 mg/L leupeptin in 0.01M phosphate buffer saline (PBS, pH 7.2), and centrifuged for 20 min at 4℃. The supernatant was then collected and total protein was determined by Bradford method. The levels of pro-inflammatory cytokines were quantified using specific ELISA kits according to the manufacturer's instructions (TNF-α from Diaclone Research, Besançon, France; IL-1β and IL-6 from Biosource Europe SA, Nivelles, Belgium). The results were expressed as pg of cytokine per 100 mg of tissue.

Quantification of survival and apoptotic neuronal numbers

The number of survival and apoptotic neurons labeled by HE and TUNEL staining, respectively, was counted by a blinded observer (L-Y Chen) with the aid of ZEISS microscope (Axioplane 2, Carl Zeiss MicroImaging GmbH, Hamburg, Germany). The number of survival neurons was counted by using the measuring grid placed on sections processed for HE staining. The density of apoptotic neurons was expressed as the number of TUNEL-labeled neurons per square millimeter of the hippocampal regions.

Computerized image analysis

The general approach for all computerized image analysis was similar to our previous studies [43-45]. The COX staining was assessed in sections processed for COX histochemistry. The staining intensity was quantified with a computer based image analysis system (MGDS) along with the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). A digital camera mounted on the ZEISS microscope (Axioplane 2, Carl Zeiss MicroImaging GmbH, Hamburg, Germany) imaged sections at 50× magnification in bright field and displayed them on a high-resolution monitor. All densitometric readings taken from all hippocampal neurons in each section were then combined and averaged to obtain the total OD (TOD) of each section. The background staining (BOD) of each section was measured by averaging 10 random rectangles

(area of rectangle = 150 μm^2) of the lateral ventricular space adjoining the hippocampus. True OD for each section was then expressed by subtracting the BOD from TOD, so that each measurement was made in an unbiased way to correct for background. All images were captured on the same day by the same experimenter to maintain the uniform settings adjusted at the beginning of capturing. As the actual amount of reaction product deposited in a tissue section as a result of enzyme activity is influenced by a variety of factors, all parameters were carefully controlled following the recommended procedures for gray level adjustment, histogram stretch and minimal OD [46].

Statistical analysis

For TOF-SIMS analysis, spectral intensity detected from each section was normalized to the ion intensity of paraformaldehyde (serve as base line = 100 %) and expressed as percentage above the base line [41]. All quantitative data acquired from spectrometric, biochemical, histological, and pathological assessments in sham-operated and bacterial exposed animals, with or without melatonin treatment, were subjected to a two-way ANOVA test. Where the effects of the experiment were found to be statistically significant, the Bonferroni *post hoc* test was used to determine which differences are statistically significant. The difference was considered significant if $P<0.05$.

RESULTS

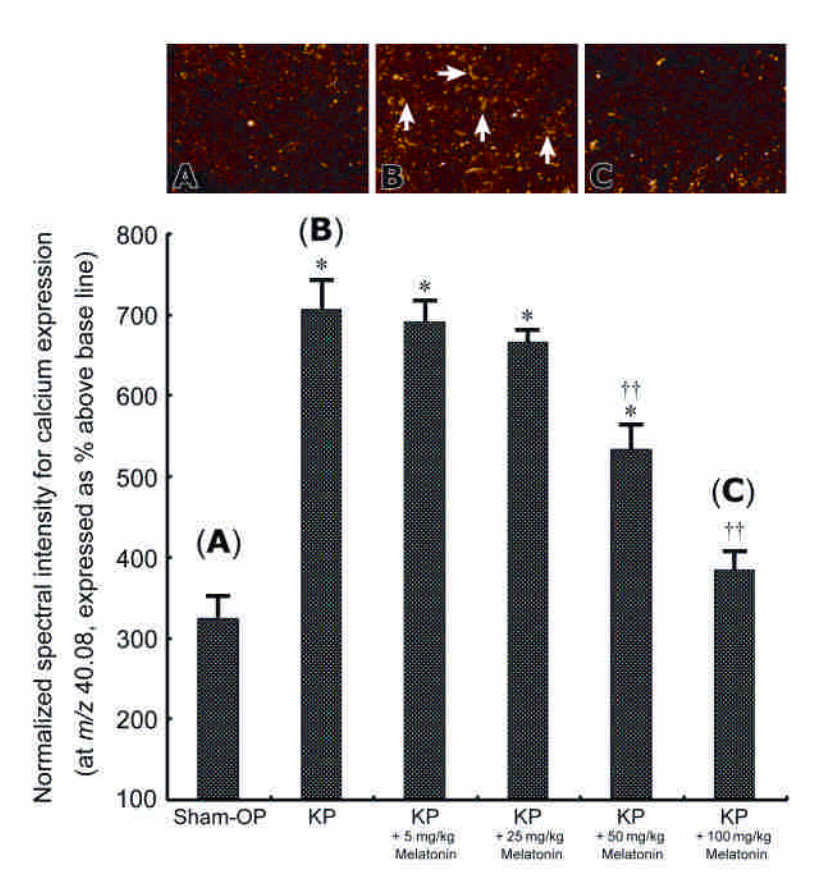


Fig. 1 Molecular imaging (A-C) and histogram showing the *in vivo* calcium ion expression and normalized spectral intensity of calcium in sham-operated, *Klebsiella pneumoniae* (KP) infected and KP infected with different doses of melatonin treatment rats. Note that in sham-operated rats, nearly none or only a few microglia with weak calcium expression were detected in hippocampus (A). Following KP infection, numerous microglia with strong calcium expressions (arrows) were found in hippocampal regions (B). However, in animals subjected to KP infection followed by different doses of melatonin, the expression of glial calcium intensity was gradually decreased with maximal change observed in animals given the dose of 100 mg/kg (C). Similar findings were also obtained from measurement of normalized spectral intensity in which melatonin significantly reduced the glial calcium intensity in hippocampus induced by KP infection.

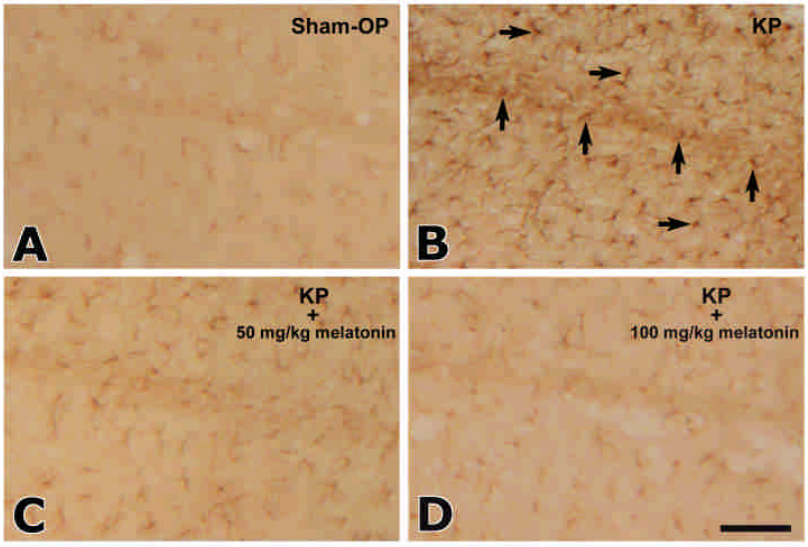
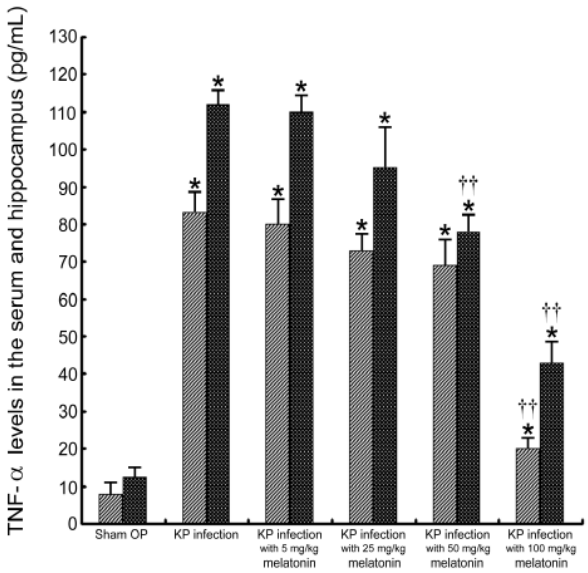
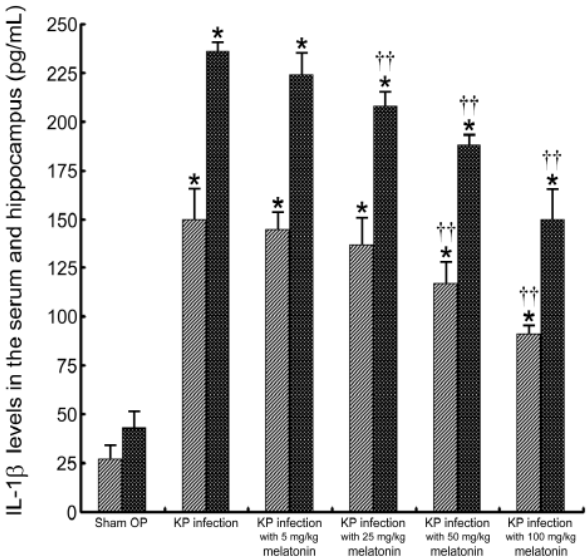


Fig. 2 Photomicrographs showing the extent of microglia activation (as demonstrated by GSA-IB4 histochemistry) in hippocampus of sham-operated, *Klebsiella pneumoniae* (KP) infected and KP infected with different doses of melatonin treatment rats. Note that in sham-operated rats, nearly no microglia was found in hippocampus (A). Following KP infection, a large number of microglia (arrows) was detected in hippocampal regions (B). However, in animals subjected to KP infection followed by different doses of melatonin, the number of microglia was significantly reduced (C) in which the maximal reduction was observed in animals given the dose of 100 mg/kg (D). Scale bar = 200 μm.


TNF- α levels in the serum and hippocampus (pg/mL)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Sham OP
KP infection
KP infection with 5 mg/kg melatonin
KP infection with 25 mg/kg melatonin
KP infection with 50 mg/kg melatonin
KP infection with 100 mg/kg melatonin


IL-1β levels in the serum and hippocampus (pg/mL)
0
25
50
75
100
125
150
175
200
225
250
Sham OP
KP infection
KP infection with 5 mg/kg melatonin
KP infection with 25 mg/kg melatonin
KP infection with 50 mg/kg melatonin
KP infection with 100 mg/kg melatonin

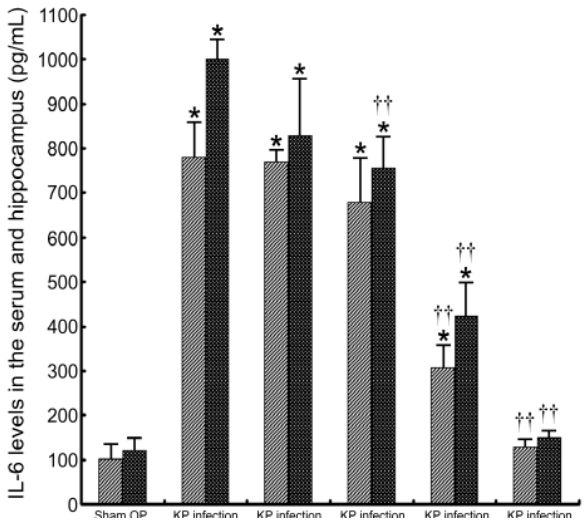

IL-6 levels in the serum and hippocampus (pg/mL)
0
100
200
300
400
500
600
700
800
900
1000
1100
Sham OP
KP infection
KP infection
KP infection
KP infection
KP infection

Fig. 3 Histograms showing the pro-inflammatory cytokine expression in serum and hippocampus of sham-operated, *Klebsiella pneumoniae* (KP) infected and KP infected with different doses of melatonin treatment rats. Note that in sham-operated rats, only small amount of pro-inflammatory cytokine (TNF-α, IL-1β, and IL-6) was detected in serum and hippocampus. Following KP infection, all examined pro-inflammatory cytokine levels were drastically increased in both serum and hippocampal regions. However, in animals subjected to KP infection followed by different doses of melatonin, the concentration of both serum and hippocampal pro-inflammatory cytokines were significantly decreased with the maximal change observed in the dose given at 100 mg/kg. ▒ : serum pro-inflammatory cytokine levels; ▓ : hippocampal pro-inflammatory cytokine levels.

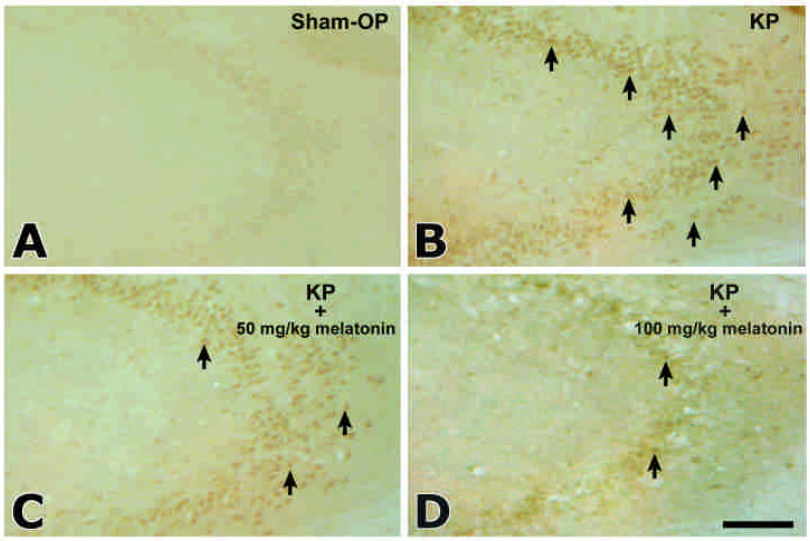


Fig. 4 Photomicrographs showing the neuronal apoptosis (as demonstrated by TUNEL staining) in hippocampus of sham-operated, *Klebsiella pneumoniae* (KP) infected and KP infected with different doses of melatonin treatment rats. Note that in sham-operated rats, no apoptotic neurons were identified in hippocampus (A). Following KP infection, a variety of apoptotic neurons with positive TUNEL staining (arrows) were detected in hippocampal region (B). However, in animals subjected to KP infection followed by different doses of melatonin, the number of apoptotic neurons was gradually decreased (C) in which only a few cells with positive TUNEL staining were observed in animals given the dose of 100 mg/kg (D). Scale bar = 200 μm.

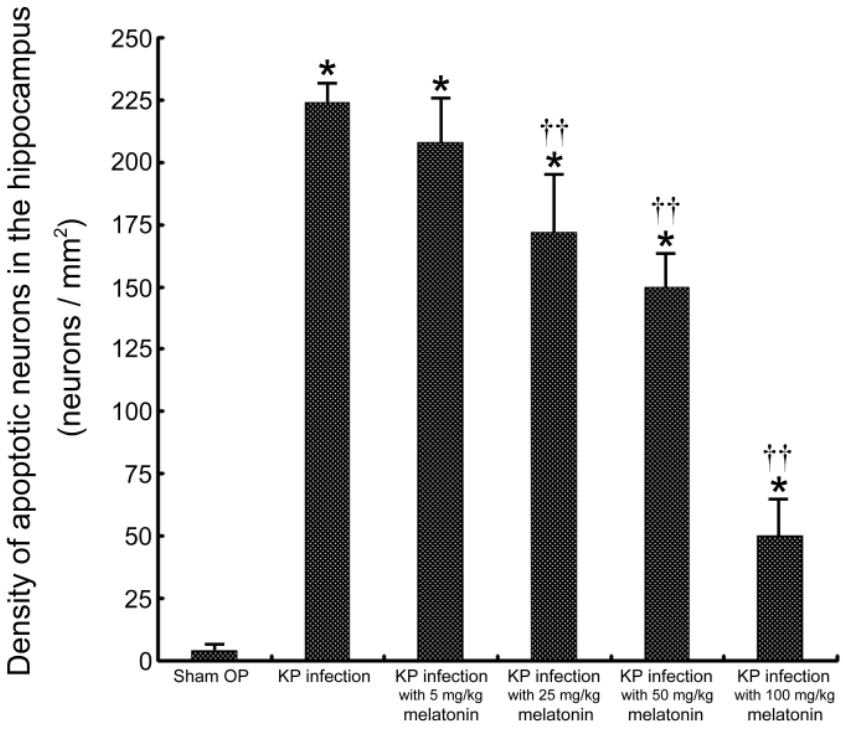


Fig. 5 Histogram showing the density of apoptotic neurons in hippocampus of sham-operated, *Klebsiella pneumoniae* (KP) infected and KP infected with different doses of melatonin treatment rats. Note that in KP infected rats, a large number of apoptotic neurons were detected in hippocampal regions. However, in animals subjected to KP infection followed by different doses of melatonin, the density of apoptotic neurons was significantly decreased with the maximal decrease observed in animals given the dose of 100 mg/kg.

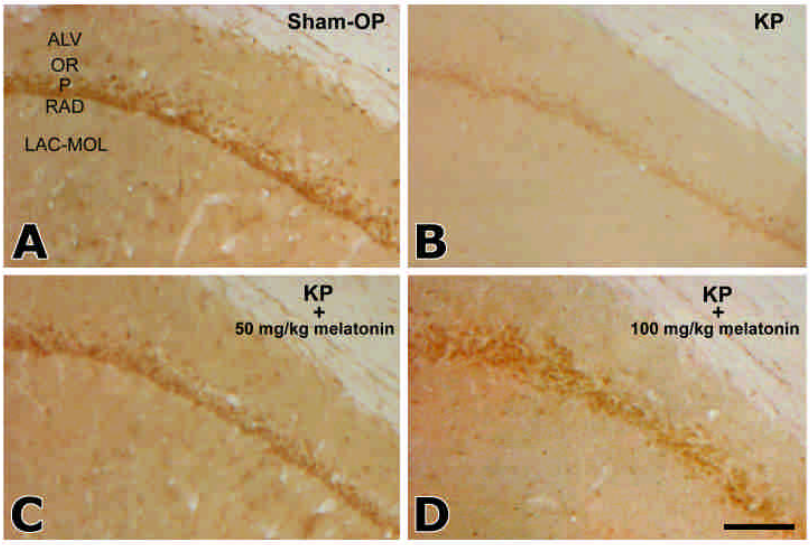


Fig. 6 Photomicrographs showing the neuronal activity [as demonstrated by cytochrome oxidase (COX) histochemistry] in hippocampus of sham-operated, *Klebsiella pneumoniae* (KP) infected and KP infected with different doses of melatonin treatment rats. Note that in sham-operated rats, a variety of neurons with high neuronal activities (strong COX staining intensities) were observed in hippocampus (A). Following KP infection, the staining intensities of COX reaction was drastically reduced in hippocampal regions (B). However, in animals subjected to KP infection followed by different doses of melatonin, the neuronal activities were gradually recovered (C) in which nearly all hippocampal neurons were stained with mild to strong COX reactivity as observed in animals given the dose of 100 mg/kg (D). ALV: alveus; OR: stratum oriens; P: pyramidal cell layer; RAD: stratum radiatum; LAC-MOL: stratum lacunosum-moleculare. Scale bar = 200 μm.

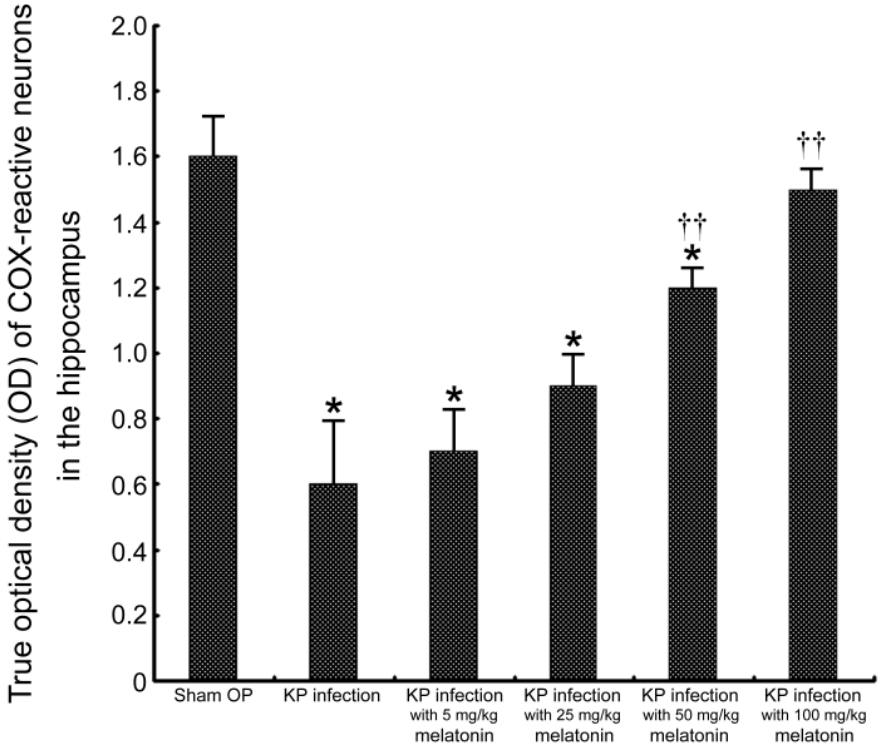


Fig. 7 Histogram showing the true optical density (OD) of cytochrome oxidase (COX) reactivity in hippocampus of sham-operated, *Klebsiella pneumoniae* (KP) infected and KP infected with different doses of melatonin treatment rats. Note that in KP infected rats, the true OD of COX reactivity was drastically decreased in hippocampal regions. However, in animals subjected to KP infection followed by different doses of melatonin, the true OD of COX reactivity was greatly preserved with the maximal change observed in animals given the dose of 100 mg/kg.

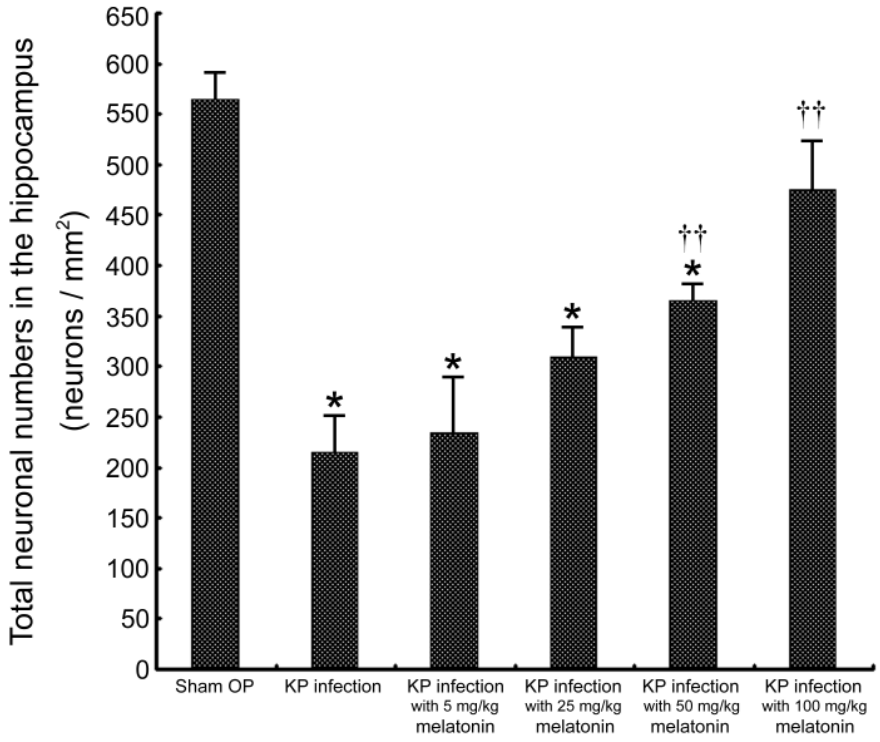


Fig. 8 Histogram showing the total neuronal number in hippocampus of sham-operated, *Klebsiella pneumoniae* (KP) infected and KP infected with different doses of melatonin treatment rats. Note that KP infection would induce lots of neuronal death in hippocampal regions. However, in animals subjected to KP infection followed by different doses of melatonin, the total neuronal number in hippocampus was successfully preserved in which the neuro-protective effect of melatonin was most evident in animals given the dose of 100 mg/kg.

Table 1 Number of leukocyte counts (cells/μL) in the cerebrospinal fluid (CSF) of *Klebsiella pneumoniae* (KP) infected rats with or without melatonin treatment (24 h following the infection)

Experimental groups	Leukocyte counts (cells/μL)
KP infection	2674 ± 945
KP infection with melatonin	
5 mg/kg	2388 ± 948
25 mg/kg	2256 ± 973
50 mg/kg	1978 ± 828
100 mg/kg	1203 ± 520 *

* $P<0.05$ as compared to the KP infection values.

ACKNOWLEDGMENTS

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