Original Research

Vitamin B6 prevents cognitive impairment in experimental pneumococcal meningitis

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Abstract

Streptococcus pneumoniae is the relevant cause of bacterial meningitis, with a high-mortality rate and long-term neurological sequelae, affecting up to 50% of survivors. Pneumococcal compounds are pro-inflammatory mediators that induce an innate immune response and tryptophan degradation through the kynurenine pathway. Vitamin B6 acts as a cofactor at the active sites of enzymes that catalyze a great number of reactions involved in the metabolism of tryptophan, preventing the accumulation of neurotoxic intermediates. In the present study, we evaluated the effects of vitamin B6 on memory and on brain-derived neurotrophic factor (BDNF) expression in the brain of adult Wistar rats subjected to pneumococcal meningitis. The animals received either 10 μL of artificial cerebral spinal fluid (CSF) or an equivalent volume of *S. pneumoniae* suspension. The animals were divided into four groups: control, control treated with vitamin B6, meningitis, and meningitis treated with vitamin B6. Ten days after induction, the animals were subjected to behavioral tests: open-field task and step-down inhibitory avoidance task. In the open-field task, there was a significant reduction in both crossing and rearing in the control group, control/B6 group, and meningitis/B6 group compared with the training session, demonstrating habituation memory. However, the meningitis group showed no difference in motor and exploratory activity between training and test sessions, demonstrating memory impairment. In the step-down inhibitory avoidance task, there was a difference between training and test sessions in the control group, control/B6 group, and meningitis/B6 group, demonstrating aversive memory. In the meningitis group, there was no difference between training and test sessions, demonstrating impairment of aversive memory. In the hippocampus, BDNF expression decreased in the meningitis group when compared to the control group; however, adjuvant treatment with vitamin B6 increased BDNF expression in the meningitis group. Thus, vitamin B6 attenuated the memory impairment in animals subjected to pneumococcal meningitis.

Keywords: Pneumococcal meningitis, vitamin B6, memory, BDNF

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Introduction

Streptococcus pneumoniae is the relevant cause of bacterial meningitis with a high-mortality rate and long-term neurological sequelae in adults,[1] affecting up to 50% of survivors.[2] Pneumococcal virulence factor is released through bacterial autolysis by *N*-acetyl-muramyl alanine amidase. These virulence factors are pro-inflammatory mediators that induce an innate immune response through activating the nuclear factor kappa B and subsequently triggering the production of pro-inflammatory cytokines, chemokines, and the expression of co-stimulatory molecules.[3,4] This pro-inflammatory environment induces tryptophan degradation through the kynurenine pathway, which is the major catabolic route of this essential amino acid.[5] Kynurenine is

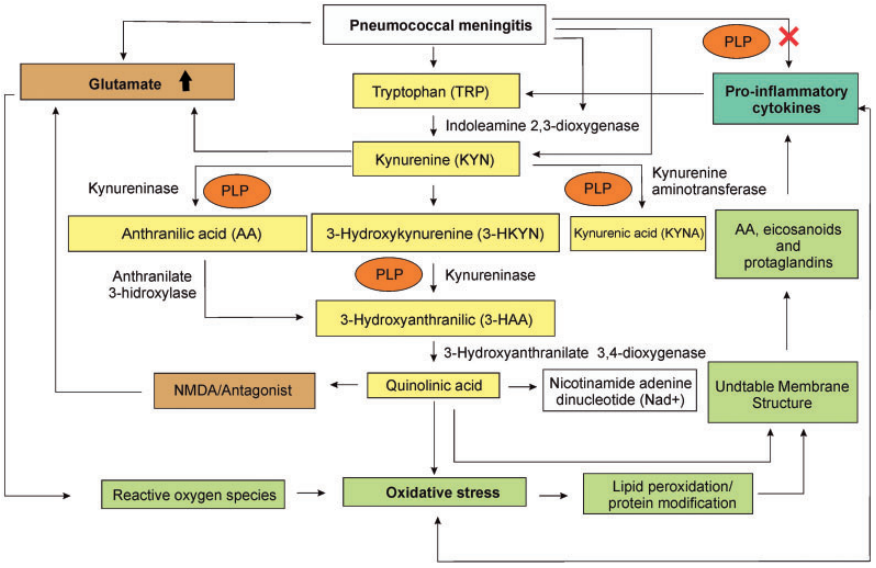

Figure 1 Pneumococcal meningitis: kynurenine pathway, oxidative stress, pro-inflammatory cytokines production, and glutamate. PLP: pyridoxal-5′-phosphate. (A color version of this figure is available in the online journal.)

then metabolized in a sequence of new compounds that have neurotoxic properties. The neurotoxic mechanism involves generation of reactive oxygen intermediates, depletion of endogenous antioxidants, and lipid peroxidation.[6] Another metabolite, quinolinic acid, acts as a neurotoxin, gliotoxin, pro-inflammatory mediator, and pro-oxidant molecule[7] (Figure 1). Pneumococcal meningitis also causes accumulation of neurotoxic kynurenine metabolites in the cortex and the hippocampus.[8]

Vitamin B6 acts as a cofactor in the metabolism of tryptophan through two enzymes, kynurenine aminotransferase and kynureninase. Thus, vitamin B6 plays a role in preventing the accumulation of neurotoxic intermediates produced during tryptophan metabolism.[9,10] Vitamin B6 has also been shown to increase the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus.[11] Furthermore, this neurotrophin is involved in neurogenesis, protects against multiple forms of brain injury in pneumococcal meningitis,[12] blocks caspase-3 activation,[13] and reduces apoptosis in animal models of meningitis.[12,14] Furthermore, it has been well established that pneumococcal meningitis survivors exhibit long-term cognitive impairment. Previously, we had determined which animals presented learning and memory impairment after pneumococcal meningitis induction.[15] In the present study, we evaluated the effects of vitamin B6 on memory and on BDNF expression in the brain of Wistar rats subjected to pneumococcal meningitis.

Experimental procedure

Infecting organism

S. pneumoniae (serotype 3) was cultured overnight in 10 mL of Todd Hewitt broth and then diluted in fresh medium and grown to the logarithmic phase. This culture was centrifuged for 10 min at 5000 × g and resuspended in sterile saline at a concentration of 5×10^9 cfu/mL. The bacterial numbers of the inoculum were confirmed by quantitative cultures.[16,17]

Animal model of meningitis

Adult male Wistar rats (250–350 g body weight) from our breeding colony were used for the experiments. All procedures were approved by the Animal Care and Experimentation Committee of UNESC/121/2012, Brazil. All surgical procedures and bacterial inoculations were performed under anesthesia, consisting of an intraperitoneal administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and acepromazine (0.16 mg/kg).[16,18] Rats underwent a cisterna magna tap with a 23-gauge needle. The animals received either 10 µL of artificial CSF or an equivalent volume of *S. pneumoniae* suspension. At the time of inoculation, animals received fluid replacement and were subsequently returned to their cages.[18,19]

Organization of the experimental groups

The animals were divided into four groups: control, control treated with vitamin B6, meningitis, and meningitis treated with vitamin B6 (20 animals per group). At the time of infection, animals ($n = 40$) received 360 µL of vitamin B6 subcutaneously (s.c.) (600 mg/kg; Sigma-Aldrich, Germany). Placebo-treated animals ($n = 40$) were injected with 360 µL of 0.85% NaCl. Eighteen hours after meningitis induction, all animals were treated with ceftriaxone (100 mg/kg body weight given i.p., during 7 days, twice daily, Roche Pharma, Rocephin®, Brazil) and a second and final dose of vitamin B6 or 0.85% NaCl.[20] At the same time point, infection was documented by quantitative culture of 5 µL of CSF. After 10 days, the animals were free from infection. During this period, we performed blood cultures that were all negative, and the animals were subjected to a behavioral task. Afterward, the animals were sacrificed, and the cortex

and hippocampus were removed for the determination of BDNF expression.

Behavioral task

Ten days after pneumococcal meningitis induction, the animals were randomized and subjected to behavioral tests: an open-field task and a step-down inhibitory avoidance task. After the behavioral tasks, the animals were killed by decapitation.

Open-field task. Behavior was assessed in an open-field apparatus to evaluate both locomotor and exploratory activities. This apparatus is a 40×60-cm open field surrounded by 50-cm high walls made of brown plywood with a front glass wall. Black lines divide the floor of the open field into nine rectangles. Animals were gently placed in the left rear quadrant and were allowed to explore the arena for 5 min. The number of crossings (i.e. the number of times that the animals crossed the black lines, which assesses locomotor activity) and rearing movements (i.e. the exploration behavior observed in rats subjected to a new environment) were measured. The same person, who was blind to group treatment, performed all behavioral testing by manual analyses.[21]

Step-down inhibitory avoidance task

The apparatus and procedures have been described in previous reports.[22,23] Briefly, the training apparatus was a $50 \times 25 \times 25$-cm acrylic box (Albarsch, Porto Alegre, Brazil) whose floor consisted of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7-cm wide, 2.5-cm high platform was placed on the floor of the box, against the left wall. In the training trial, animals were placed on the platform, and their latency to step down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, the animals received a 0.4-mA, 2.0-s foot shock and were returned to their home cage. A retention test trial was performed 1.5 h (short-term memory) and 24 h after training (long-term memory). The retention test trial was procedurally identical to the training trial, except that no foot shock was presented. The retention-test step-down latency (maximum, 180 s) was used as a measure of inhibitory avoidance retention.[24,25]

Assessment of BDNF expression

The measurement of BDNF levels was performed as previously described.[26] BDNF serum levels were measured with sandwich-ELISA, using a commercial kit according to the manufacturer's instructions (Millipore, USA). Briefly, microtiter plates (96-well flat-bottom) were coated overnight at 4°C with the samples diluted 1:100 in sample diluent and the standard curve ranged from 7.8 to 500 pg/mL of BDNF. Plates were then washed four times with wash buffer, and biotinylated mouse anti-human BDNF monoclonal antibody (diluted 1:1000 in sample diluent) was added, followed by incubation for 3 h at room temperature. After washing, a second incubation with streptavidin-horseradish peroxidase conjugate solution (diluted 1:1000) was carried out for 1 h at room temperature. After the addition of substrate and stop solution, the amount of BDNF was determined (absorbance set in 450 nm). The standard curve demonstrates a direct relationship between optical density and BDNF concentration. Total protein was measured by Bradford's method (samples diluted 1:200) using bovine serum albumin (BSA) as a standard.

Statistics

Data from the habituation to the open-field task are reported as the mean ± SEM and were analyzed by ANOVA. Differences within groups between training and testing were assessed by the paired Student's *t*-test. Because dependent variable measured is not interval scale, data from the inhibitory avoidance task are reported as median and interquartile ranges. In addition, comparisons among groups were performed using Mann–Whitney *U*-tests. Data within individual groups were analyzed by Wilcoxon tests. P values < 0.05 were considered statistically significant. All data from BDNF were reported as the mean ± SEM and were analyzed by two-way ANOVA, followed by a Bonferroni *post hoc* test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software version 20.0.

Results

In the open-field task (Figure 1), we evaluated the influence of vitamin B6 on habituation memory 10 days after the induction of pneumococcal meningitis. There were no differences in the number of crossing and rearing movements among groups in the habituation to the open-field training session, demonstrating no difference in motor and exploratory activity among groups. In the test session, there was a significant reduction in both crossings and rearing in the control group, control/B6 group, and the meningitis/B6 group when compared with the training session, demonstrating habituation memory in these groups ($P < 0.01$). However, the meningitis group showed no difference in motor and exploratory activity between training and test sessions, demonstrating habituation memory impairment in this group.

Figure 2 shows the results of a step-down inhibitory avoidance task 10 days after pneumococcal meningitis induction. There was a difference between training and test sessions in the control group, control/B6 group, and meningitis/B6 group, demonstrating aversive memory in these groups ($P < 0.05$). In the meningitis group, there was no difference between training and test sessions, demonstrating impairment of aversive memory in this group ($P > 0.05$).

Figure 3 shows BDNF expression in the hippocampus and frontal cortex 10 days after pneumococcal meningitis induction. In the hippocampus, BDNF expression decreased in the meningitis group compared to the control groups ($F_{(1,37)} = 15.03$, $P < 0.001$). Adjuvant treatment with vitamin B6 increased BDNF expression in the meningitis

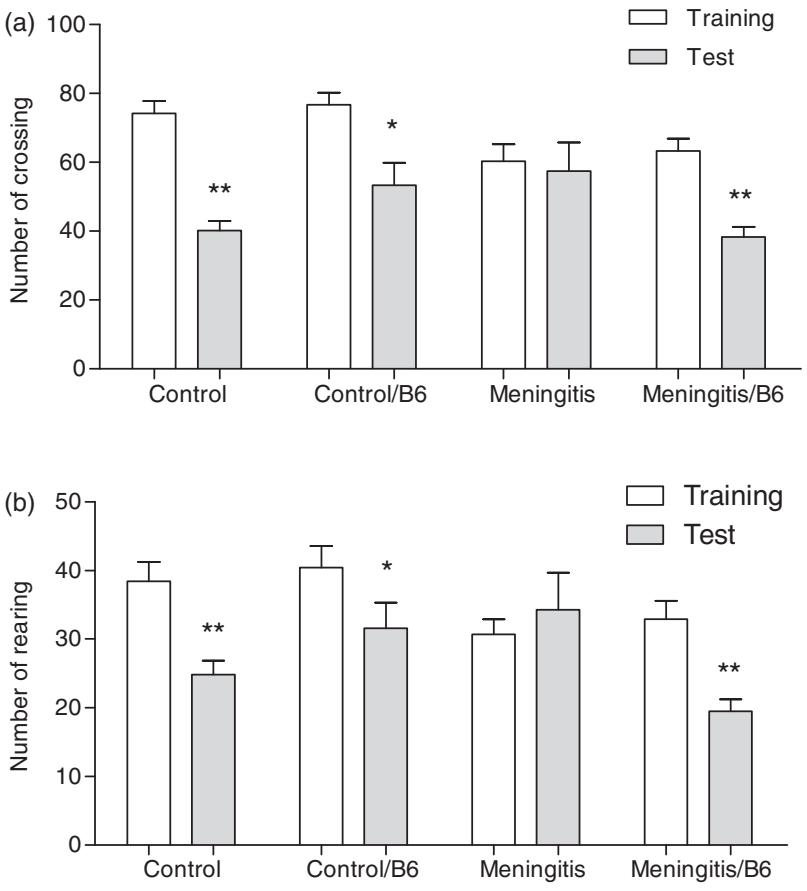

Figure 2 Open-field test 10 days after pneumococcal meningitis induction. The number of crossings (a) and the number of rearings (b) are shown. Data are reported as the mean ± SEM in training and test sessions, $n = 10$ per group and were analysed by a paired Student's *t*-test, ANOVA, and a Tukey's *post hoc* test. In all comparisons, $P < 0.05$ indicated statistical significance. Values $^{**}P < 0.01$ and $^{*}P < 0.05$ indicate differences between training and test sessions for the same group (paired *t*-test)

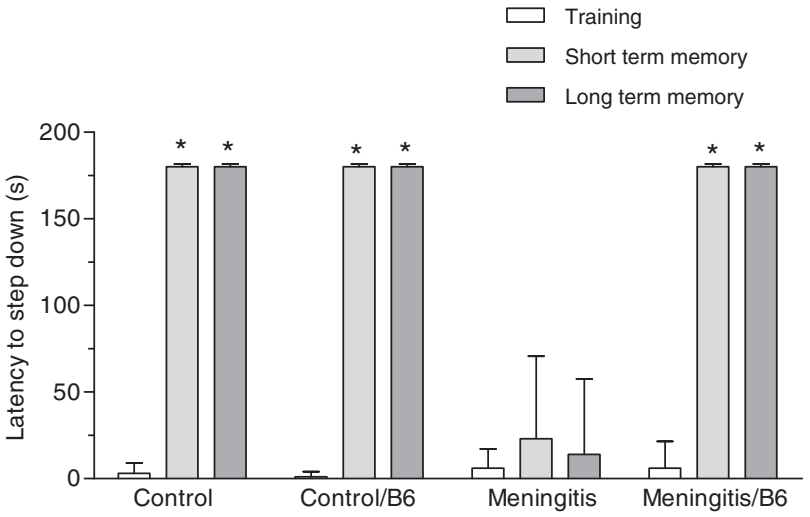

Figure 3 Latency to step-down in the inhibitory avoidance task 10 days after pneumococcal meningitis induction. Latency to step down was recorded during training at 1.5 h (short-term memory, STM) and 24 h (long-term memory, LTM) for the sham and meningitis groups. Data are reported as median and interquartile ranges, and comparisons among groups were performed using Mann–Whitney *U*-test. The within-individual groups were analysed by Wilcoxon's tests, $n = 10$ animals per group. $^{**}P < 0.01$ and $^{*}P < 0.05$ indicate differences between training and test sessions for the same group (Wilcoxon test)

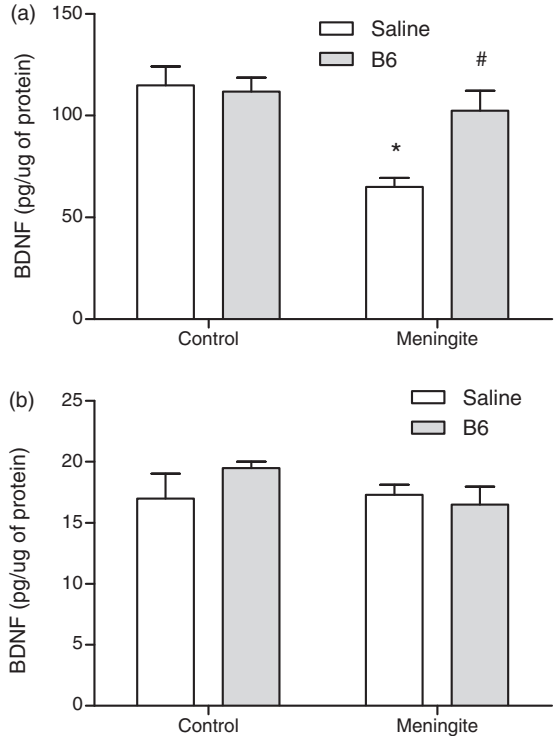

Figure 4 BDNF expression in the hippocampus and frontal cortex 10 days after pneumococcal meningitis induction. The results are shown for the hippocampus (a) and the frontal cortex (b). BDNF levels were assessed by ELISA, and the results are presented in picagram per 100 mg of tissue, $n = 10$ per group. The data are reported as the mean ± SEM and were analyzed with two-way ANOVA followed by a Bonferroni *post hoc* test. #Symbols indicate a statistically significant difference between the meningitis group and the meningitis/vitamin B6 group ($P < 0.05$). *Symbols indicate a statistically significant difference between the meningitis group and the control groups ($P < 0.01$)

group ($F_{(1,37)} = 9.855$, $P < 0.00001$). There was no change in frontal cortex expression of BDNF.

Discussion

In the present study, we demonstrated the influence of vitamin B6 on learning, memory, and BDNF expression in experimental pneumococcal meningitis. Several pneumococcal compounds are pro-inflammatory mediators inducing an innate immune response.[3] These pro-inflammatory cytokines activate the metabolic enzyme indoleamine 2,3-dioxygenase,[27] which is widely expressed in all tissues including epithelial cells, macrophages, and dendritic cells.[28] Indoleamine 2,3-dioxygenase in microglia catabolizes the amino acid L-tryptophan to kynurenine.[29,30] Kynurenine is then metabolized in a succession of novel compounds that have neurotoxic properties.[8,31,32] The kynurenine pathway and one of its end-products, the excitotoxin quinolinic acid, are implicated in the pathogenesis of some neuroinflammatory brain diseases.[33] Various regions of the rat brain have shown differences in vulnerability to the neurotoxic effects of infusion of quinolinic acid, with the hippocampus exhibiting the greatest susceptibility.[34,35] Pneumococcal meningitis also leads to the accumulation of kynurenine metabolites in the brain with a positive correlation between the concentration of kynurenine metabolites and the extent of apoptosis damage in the hippocampus.[8] Previous studies have shown that a low concentration of vitamin B6 in plasma is related with increased levels of pro-inflammatory cytokines.[36] Supplementation with vitamin B6 suppressed pro-inflammatory cytokines in patients with rheumatoid arthritis.[37] In the present study, we showed that, in animals subjected to pneumococcal meningitis, adjuvant treatment with vitamin B6 prevented cognitive impairment and increased BDNF expression in the hippocampus. In another study, indoleamine 2,3-dioxygenase inhibitor also prevented cognitive impairment through interruption of the kynurenine pathway.[38] Zysset-Burri et al.[11] demonstrated that vitamin B6 reduced hippocampal apoptosis, prevented cellular energy storage through the increased of NAD+ levels, and up-regulated BDNF gene expression level 24 h after infection in infant model of pneumococcal meningitis. Thus, the up-regulated expression of BDNF could have had an important role in the diminished apoptosis observed in this experiment. In our study, 10 days after pneumococcal meningitis induction, BDNF levels remained elevated in the hippocampus and lack of cognitive impairment.

Conclusion

Adjuvant treatment with vitamin B6 exerted neuroprotective effects through increased BDNF expression in the hippocampus and attenuation of memory impairment in animals subjected to pneumococcal meningitis.

Author contributions: All authors participated in the design and interpretation of the studies, analysis of the data, and review of the manuscript; JSG, LRS, RAC, PF, CG, and FK conducted the experiments; JQ, JB, and DD were responsible for the behavioral tests; LKJ did the statistical tests, and TB wrote the manuscript.

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