


# Vitamin B<sub>6</sub> Reduces Neurochemical and Long-Term Cognitive Alterations After Polymicrobial Sepsis: Involvement of the Kynurenine Pathway Modulation

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**Abstract** Neurological dysfunction as a result of neuroinflammation has been reported in sepsis and cause high mortality. High levels of cytokines stimulate the formation of neurotoxic metabolites by kynurenine (KYN) pathway. Vitamin B<sub>6</sub> (vit B<sub>6</sub>) has anti-inflammatory and antioxidant properties and also acts as a cofactor for enzymes of the KYN pathway. Thus, by using a relevant animal model of polymicrobial sepsis, we studied the effect of vit B<sub>6</sub> on the KYN pathway, acute neurochemical and neuroinflammatory parameters, and cognitive dysfunction in rats. Male Wistar rats (250–300 g) were submitted to cecal ligation and perforation (CLP) and divided into sham + saline, sham + vit B<sub>6</sub>, CLP + saline, and CLP + vit B<sub>6</sub> (600 mg/kg, s.c.) groups. Twenty-four hours later, the prefrontal cortex and hippocampus were removed for neurochemical and neuroinflammatory analyses. Animals were followed for 10 days to determine survival rate, when cognitive function

was assessed by behavioral tests. Vitamin B<sub>6</sub> interfered in the activation of kynurenine pathway, which led to an improvement in neurochemical and neuroinflammatory parameters and, consequently, in the cognitive functions of septic animals. Thus, the results indicate that vit B<sub>6</sub> exerts neuroprotective effects in acute and late consequences after sepsis.

**Keywords** Sepsis · Vitamin B<sub>6</sub> · Brain damage · Tryptophan · Oxidative stress · Neuroinflammation

## Introduction

Sepsis is a common clinical syndrome in intensive care units (ICUs), with mortality rates that reach 60% [1]. The development of organ dysfunction is a complication that

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contributes to increased mortality in sepsis, and the brain is particularly affected [2, 3].

In sepsis, the acute brain dysfunction is known as sepsis-associated encephalopathy (SAE), which manifests itself in up to 70% of septic patients and is associated with a worst prognosis [4]. Neuroinflammation, ischemia, and neurotoxicity are the main processes implicated in SAE [5–7]. Particularly, neuroinflammation increases the metabolic and bioenergetic demands, which may result in oxidative stress and mitochondrial dysfunction [8].

Indeed, early mitochondrial dysfunction has been detected in the brain of septic animals, resulting in the production of reactive oxygen (ROS) and nitrogen (RNS) species. The production of reactive species stimulates a pro-apoptotic state that affects glial cells, neurons, and blood brain barrier (BBB) structure [9].

One pathway that may be related to the progression of neurological damage in sepsis is the kynurenine (KYN) pathway. Critically ill patients may exhibit elevated levels of KYN in the expense of tryptophan (TRP) plasmatic level depletion [10, 11]. During active inflammation, indoleamine 2,3-dioxygenase (IDO) is activated in extrahepatic tissues to convert TRP into KYN [12]. This is followed by the synthesis of enzymes that regulate the generation of neurotoxic metabolites, such as 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid. The neurotoxicity mediated by these metabolites is related to ROS formation.

Vitamin B<sub>6</sub> (vit B<sub>6</sub>) acts as cofactor of these enzymes and could modulate the production of these metabolic intermediates [13]. In addition, vit B<sub>6</sub> performs important functions, including the modulation of inflammatory response and oxidative stress [14]. Reduced plasmatic level of vit B<sub>6</sub> is associated with oxidative stress, immune system disorders, and disturbances in glucose metabolism [15–17]. On the other hand, adequate levels of vit B<sub>6</sub> are positively correlated with increased levels of antioxidant enzymes [18, 19].

These evidences point out to a possible positive role of vit B<sub>6</sub> in the genesis of brain dysfunction in sepsis. Thus, the purpose of this study was to examine the effect of vit B<sub>6</sub> on survival, kynurenine pathway activation, acute neuroinflammation, and long-term cognitive dysfunction in a preclinical model of sepsis.

## Materials and Methods

### Animals

Adult male *Wistar* rats, approximately 60 days old (weighing 250–350 g), from breeding colonies maintained at the Universidade do Sul de Santa Catarina were used. The animals were housed five per cage under controlled conditions of temperature ( $22 \pm 1$  °C), relatively

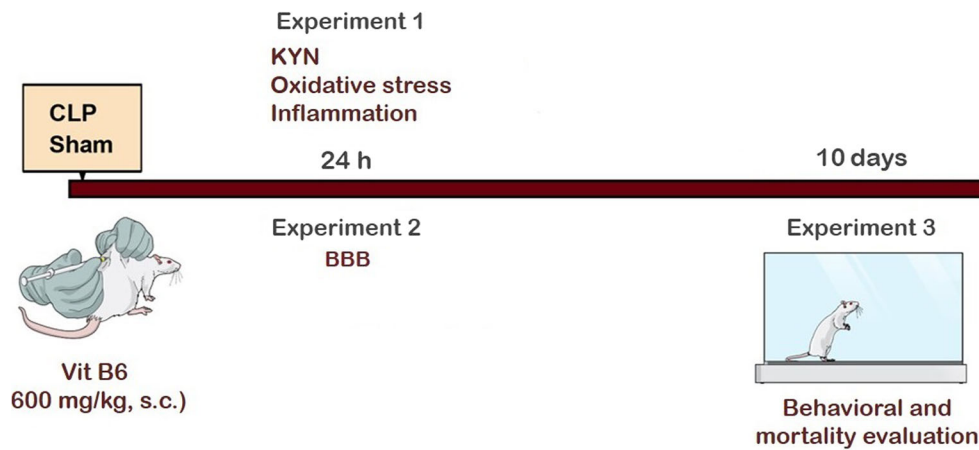
humidity (45–55%), and day/light cycle (12:12-h, light on at 0600 hours). Rat chow (standard diet for laboratory animals—NUVILAB CR-1®, Brazil) and tap water were available ad libitum. This study was approved by the Animal Research Ethic Committee of the Universidade do Sul de Santa Catarina (protocol #15.012.4.03.IV). The “Principles of Laboratory Animal Care” (NIH publication no. 80–23, revised 1996) and the “EC Directive 86/609/EEC” were followed in all experiments. All efforts were made to minimize the number of animals used and their suffering.

### Sepsis Induction—CLP Model

Rats were subjected to cecal ligation and perforation (CLP) as previously described [20]. Briefly, they were anesthetized intraperitoneally (i.p.) with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). Under aseptic conditions, a 3-cm midline laparotomy was performed to expose the cecum and adjoining intestine. The cecum was tightly ligated with a 3.0 silk suture at its base, below the ileocecal valve, and was perforated once with 14-gauge needle. The cecum was then squeezed gently to extrude a small amount of feces through the perforation site. The cecum was then returned to the peritoneal cavity, and the laparotomy was closed with 4.0 silk sutures. Animals were resuscitated with regular saline (50 mL/kg) subcutaneously (s.c.) immediately after CLP, ceftriaxone (30 mg/kg), and dipyrone (80 mg/kg) s.c. immediately after and 12 h after CLP and were returned to their cages with free access to food and water. In the sham-operated group, the rats were submitted to all surgical procedures, but the cecum was neither ligated nor perforated. To minimize variability between different experiments, the CLP procedure was always performed by the same investigators.

### Treatments and Samples Obtention

Vit B<sub>6</sub> was obtained from Fagron (São Paulo, BRA) and dissolved in saline immediately before use. Immediately after CLP surgery, rats received 600 mg/kg s.c. of vit B<sub>6</sub> or the same volume of saline [21]. Four groups ( $n = 10$  per group) were randomly divided into the following: (1) sham + saline; (2) sham + vit B<sub>6</sub>, (3) CLP + saline, and (4) CLP + vit B<sub>6</sub>. For the acute neuroinflammation assays, rats were followed during 24 h (experiments 1 and 2 such as Fig. 1). For survival analysis, animals were followed during 10 days and deaths were counted daily (experiment 3 such as Fig. 1). The long-term cognitive dysfunction was evaluated by behavioral tests initiated on day 10 (experiment 3 such as Fig. 1). After each experimental time-point, animals were subjected to a thiopental overdose (0.5 g/kg) followed by decapitation. The prefrontal cortex and hippocampus were quickly isolated and stored at  $-80$  °C for subsequent biochemical analysis [22].



**Fig. 1** Experimental design. The rats were induced to sham (control) or sepsis by CLP. Immediately after CLP surgery, rats received 600 mg/kg s.c. of vit B<sub>6</sub> or the same volume of saline. In the experiment 1, after 24 h in the prefrontal cortex and hippocampus, kynurenine, tryptophan, and

the tryptophan/kynurenine ratio, oxidative stress, and neuroinflammation were evaluated. In the experiment 2, in the 24 h, the BBB permeability was evaluated, and 10 days after CLP in the experiment 3, behavioral tests and survival was evaluated

## IDO Activation

### Sample Preparation

Samples were added with three volumes of ice cold methanol (50  $\mu$ L + 150  $\mu$ L), vortexed, and centrifuged at 14 $\times$ g for 10 min (4  $^{\circ}$ C). Hydrophobic compounds (fatty acids, proteins) were removed from the supernatant using activated C18 resin. The C18 resin was previously activated by conditioning it twice with 300  $\mu$ L of sample solvent (75% methanol) followed by centrifugation at 14 rpm for 10 min (room temperature). Activated C18 resin was added to the samples at the proportion of 1/5 of the sample mass (10 mg), vortexed, and centrifuged as above. Supernatant was transferred to a new tube and solvent was removed in a SpeedVac. Samples were reconstituted in the same volume of MilliQ water and transferred to 100  $\mu$ L polypropylene vials and placed in a cooled autosampler (5  $^{\circ}$ C) until 20  $\mu$ L was injected in the LC-MS system in the same day.

### Liquid Chromatography-Mass Spectrometry

TRP and the KYN standards were purchased from Sigma-Aldrich (St. Louis, MO) with 96% purity. Liquid chromatography-mass spectrometry (LC-MS) analyses were performed on a Nexera Ultra High Performance Liquid Chromatography (UHPLC) system (Shimadzu, Kyoto, Japan) hyphenated to a maXis ETD high-resolution Electrospray ionization quadrupole time-of-flight (ESI-QTOF) mass spectrometer (Bruker, Billerica, MA) and controlled by the Compass 1.5 software package (Bruker). Fifty microliters of standards and samples were injected into a Shimadzu Shim-Pack XR-ODSIII column (C18, 2.2  $\mu$ m, 80  $\text{\AA}$ , 2.2  $\times$  200 mm) at 30  $^{\circ}$ C under a flow rate of 200  $\mu$ L/min. Elution of TRP and KYN was achieved with the mobile phases A and B (0.1% formic acid in water resp. methanol) as

follows: 01 min 3% B, 1–17 min 30% B, 17–18 min 30, 100% B, 18–23 min 100% B.

Mass spectra were acquired in positive ion mode between  $m/z$  40,300 and at a spectra rate of 0.5 Hz. Ion source parameters were set to 500 V end plate offset, 4500 V capillary voltage, 2.0 bar nebulizer pressure, 8.0 L/min, and 200  $^{\circ}$ C dry gas flow resp. ion source temperature. Ion cooler settings were optimized for sensitivity in the mentioned  $m/z$  range using 10 mM sodium formate/acetate in 50% 2-propanol as calibrant solution. Mass calibration was achieved by initial ion source infusion of 20  $\mu$ L calibrant solution and post-acquisition recalibration of the raw data.

Standard mixtures were injected in a concentration range between 0.8 and 1000 ng/mL in phosphate-buffered saline to yield the calibration curves for each compound. Between the samples, individual standard mixtures with 100 ng/mL of each metabolite were injected to monitor the system stability. The calibration as well as the sample data were post-processed and analyzed via the QuantAnalysis software (Bruker) which extracts the high-resolution ion chromatograms (EICs) of the listed target metabolites, and integrates the chromatographic peaks in the retention time windows according to the recorded calibration curves of the respective standard compounds. The curve regression factors within the concerning concentration ranges were 0.995 or higher.

### Enzymatic Activity of IDO

The activity of IDO in the prefrontal cortex and hippocampus was determined by the ratio of TRP and KYN concentrations [23].

### BBB Permeability Analysis

The integrity of the BBB was investigated using Evans blue dye extravasation [24]. The dye was administered (2% wt/vol

in phosphate-buffered saline; PBS) intravenously (3 mL/kg) through the femoral vein. The animals were perfused 1.5 h after using normal saline (250 mL) through the left ventricle at 110 mmHg pressure until colorless perfusion fluid was obtained from the right atrium. Quantitative evaluation of BBB permeability was achieved by measuring the content of Evans blue in the hippocampus and prefrontal cortex by its fluorescence intensity (Spectramax M2 microplate reader, Molecular Devices). The results were expressed as nanogram/milliliter of brain tissue.

### Myeloperoxidase Activity

Neutrophil infiltrate in tissues was measured by myeloperoxidase (MPO) activity. Brain tissues were homogenized (50 mg/mL) in 0.5% hexadecyltrimethylammonium bromide and centrifuged at  $15,000\times g$  for 40 min. The suspension was then sonicated three times for 30 s. An aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM  $H_2O_2$ . Activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37 °C. Data was expressed as milliunits/milligram protein [25].

### Cytokine Measurements

The concentrations of tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) in brain structures were determined by a standard sandwich enzyme-linked immunosorbent assay (ELISA), with commercial available kits (R&D Systems, Minneapolis, MN). The results were expressed as picogram/milliliter.

### Measurement of Nitrite/Nitrate Concentration

Nitrite/nitrate concentration was assayed spectrophotometrically using Griess reagents [1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride in bidistilled  $H_2O$  (NED solution)] and vanadium(III) chloride as described previously [26]. A standard curve was run simultaneously with each set of samples and the optical density at 550 nm (OD550) was measured using ELISA microplate reader. Data was expressed as nanomole/milligram protein.

### Oxidative Damage Parameters

The formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction was verified as an index of oxidative damage on lipids, as previously described [27]. Briefly, the samples were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 15 min. TBARS was determined by the absorbance at 535 nm using 1,1,3,3-

tetramethoxypropane as an external standard. Results were expressed as malondialdehyde equivalents nanomole/milligram protein.

The protein oxidation was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhydrazine, as previously described [28]. Briefly, proteins were precipitated by the addition of 20% trichloroacetic acid and dissolved in dinitrophenylhydrazine, and the absorbance was read at 370 nm. The results were expressed as protein carbonylation nanomole/milligram protein.

### Antioxidant Enzymes Activity

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 32 °C [29]. Absorption was measured at 480 nm. SOD specific activity was represented as milliunits/milligram protein.

Catalase (CAT) (EC 1.11.1.6) activity was assayed by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM  $H_2O_2$ , 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and the supernatants containing 0.1–0.3 mg protein  $mL^{-1}$  [30]. The specific activity was expressed as milliunits/milligram protein.

### Mitochondrial Electron Transport Chain Enzymes Activity

After sample preparation, mitochondrial respiratory chain enzyme activities complex I was evaluated using the method previously described by measuring the NADH-dependent ferricyanide reduction rates at 420 nm [31]. The activity of complex III was measured by the decrease in absorbance of 2,6-dichloroindophenol. Reading was held in spectrophotometer at 600 nm for 5 min and the absorbance recorded every minute [32]. The complex IV activity was assayed according to the method described by [33], measured by examining the decrease in absorbance at 550 nm due to the oxidation of previously reduced cytochrome c. The activities of the mitochondrial respiratory chain complexes were expressed as nanomole/minute/milligram protein.

### Protein Determination

All results are normalized with proteins were measured by the Lowry method [34].

### Behavioral Analyses

#### *Open-Field Task*

The habituation to the open-field task evaluates motor performance in the training section and non-associative memory in

the retention test session. Habituation to an open field was carried out in a 40 × 60 cm open field surrounded by 50-cm-high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. The animals were gently placed on the left rear quadrant and left to explore the arena for 5 min (training session). Immediately following this, the animals were taken back to their home cage and 24 h later submitted again to a similar open-field session (test session). Crossing of the black lines and rearing performed in both sessions were counted. The decrease in the number of crossings and rearings between the two sessions was taken as a measure of the retention of habituation [35].

#### Object Recognition Task

This task evaluates non-aversive and non-spatial memory. The apparatus and procedures for the object recognition task have been described elsewhere [36, 37]. Briefly, the task took place in a 40 × 50 cm open field surrounded by 50-cm-high walls made of plywood with a frontal glass wall. The floor of the open field was divided into nine equal rectangles by black lines. All animals were submitted to a habituation session where they were allowed to freely explore the open field for 5 min. No objects were placed in the box during the habituation trial. Crossings of the black lines and rearings performed in this session were evaluated as locomotor and exploratory activity, respectively. At different times after habituation, training was conducted by placing individual rats for 5 min in the field, in which two identical objects (objects A1 and A2, both being cubes) were positioned in two adjacent corners, 10 cm from the walls. In a short-term recognition memory test performed 1.5 h after training, the rats explored the open field for 5 min in the presence of one familiar (A) and one novel (B, a pyramid with a square-shaped base) object. All objects had similar textures (smooth), colors (blue), and sizes (weight 150–200 g), but distinctive shapes. A recognition index calculated for each animal is reported as the ratio  $TB/(TA + TB)$  ( $TA$  = time spent exploring the familiar object A;  $TB$  = time spent exploring the novel object B). Recognition memory was evaluated as done for the short-term memory test. Exploration was defined as sniffing (exploring the object 3–5 cm away from it) or touching the object with the nose and/or forepaws [38].

#### Data Analysis

Results were analyzed by Statistical Package for the Social Sciences software (SPSS, Chicago, IL, USA). Data are expressed as mean ± S.D. Differences among experimental groups were determined by two-way analysis of variance (ANOVA), followed by Tukey or Bonferroni post hoc test, respectively. Data from behavioral analysis were expressed as median and interquartile range, and comparisons among groups

were performed using Mann-Whitney  $U$  tests, while the within-individual groups were analyzed by Wilcoxon test. The survival analysis was assessed by Kaplan-Meier and log rank test. In all comparisons, statistical significance was set at  $p < 0.05$ .

## Results

### Vit B<sub>6</sub> Decreases Disrupted Tryptophan Metabolism

Figure 2 show levels of kynurenine, tryptophan, and the tryptophan/kynurenine ratio in the hippocampus and prefrontal cortex of rats submitted to severe polymicrobial sepsis and treated with vitamin B<sub>6</sub>.

In the hippocampus, we observed that CLP + vit B<sub>6</sub> animals presented a significant increase in TRP levels (~ 50%), compared to both CLP + saline and sham + saline animals. With regards to KYN levels, we did not detect statistically significant results among groups; however, we observed a tendency in CLP + vit B<sub>6</sub> group in triplicating KYN levels, compared to CLP + saline group. Concerning the TRP/KYN ratio, although statistical analysis did not show significant differences, we can perceive that, since TRP levels increased ~ 50% and there was a threefold increase in KYN levels, the TRP/KYN of CLP + vit B<sub>6</sub> group tended to decrease (Fig. 2a).

In the prefrontal cortex, we observed that kynurenine concentrations were significantly affected by sepsis and by vit B<sub>6</sub> treatment, while tryptophan concentrations were only affected by sepsis and the TRP/KYN ratio was only interfered by vit B<sub>6</sub> treatment. A paired-group comparison detected increased concentrations of tryptophan (~ 25%) and kynurenine (~ 65%) in CLP + vit B<sub>6</sub> animals, in relation to CLP + saline rats. As a result, the TRP/KYN ratio did not vary among these groups. When we compared the CLP + vit B<sub>6</sub> and sham + saline groups, we noted that vit B<sub>6</sub> stimulated a threefold increase in the kynurenine concentrations, but also increased tryptophan concentrations (~ 60%), which explains the significant difference between the TRP/KYN ratios of these two groups (Fig. 2b).

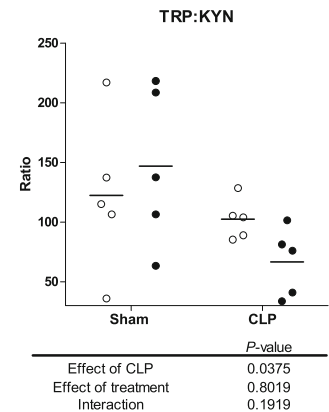
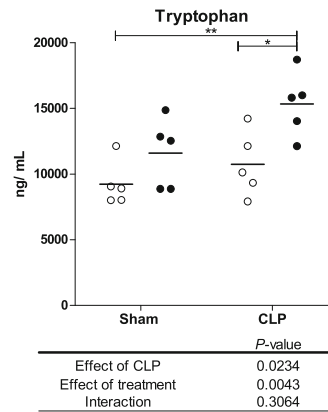
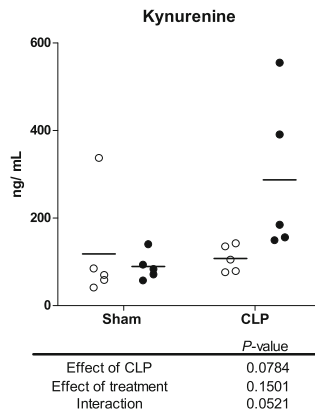
### Vit B<sub>6</sub> Decreases BBB Permeability

Figure 3 shows BBB permeability measured by Evans blue dye extravasation. BBB permeability was increased in CLP + saline group in the prefrontal cortex and hippocampus, and treatment with vit B<sub>6</sub> significantly reduced this alteration in both structures analyzed.

### Vit B<sub>6</sub> Attenuates Neuroinflammation in Sepsis

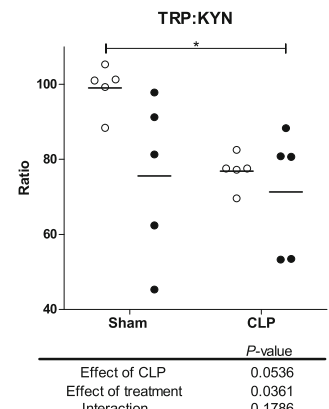
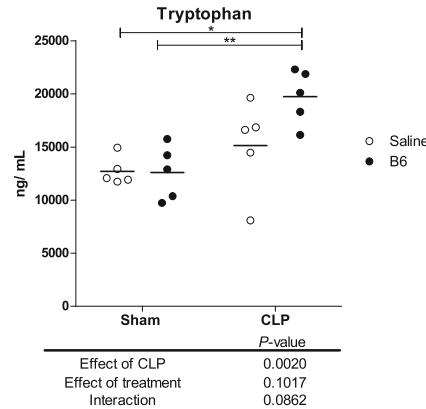
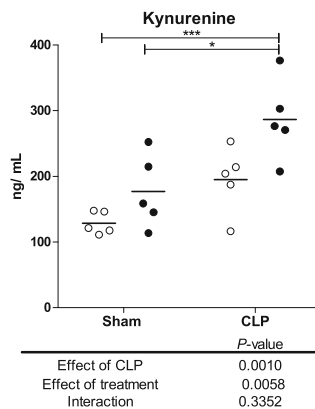
Treatment with vit B<sub>6</sub> decreased neutrophil infiltration in the hippocampus (Fig. 4a) of septic rats, compared to CLP + saline. The levels of TNF- $\alpha$  (Fig. 4b) were increased in the prefrontal cortex and hippocampus of rats 24 h after sepsis

## a Hippocampus



○ Saline  
● B6

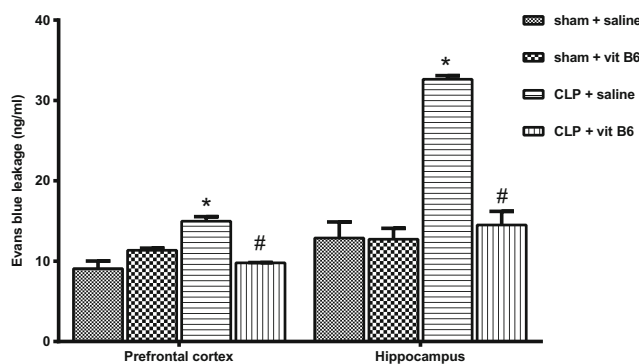
## b Cortex



**Fig. 2** Levels of kynurenine, tryptophan, and the tryptophan/kynurenine ratio in the hippocampus (a) and prefrontal cortex (b) of rats subjected to polymicrobial sepsis and treated with vit B<sub>6</sub> (600 mg/kg). Data are

expressed as mean ± standard deviation, analyzed by two-way ANOVA with post hoc Bonferroni. \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001

induction, while vit B<sub>6</sub> was effective in decreasing it in both structures. The levels of IL-1β (Fig. 4c) increased

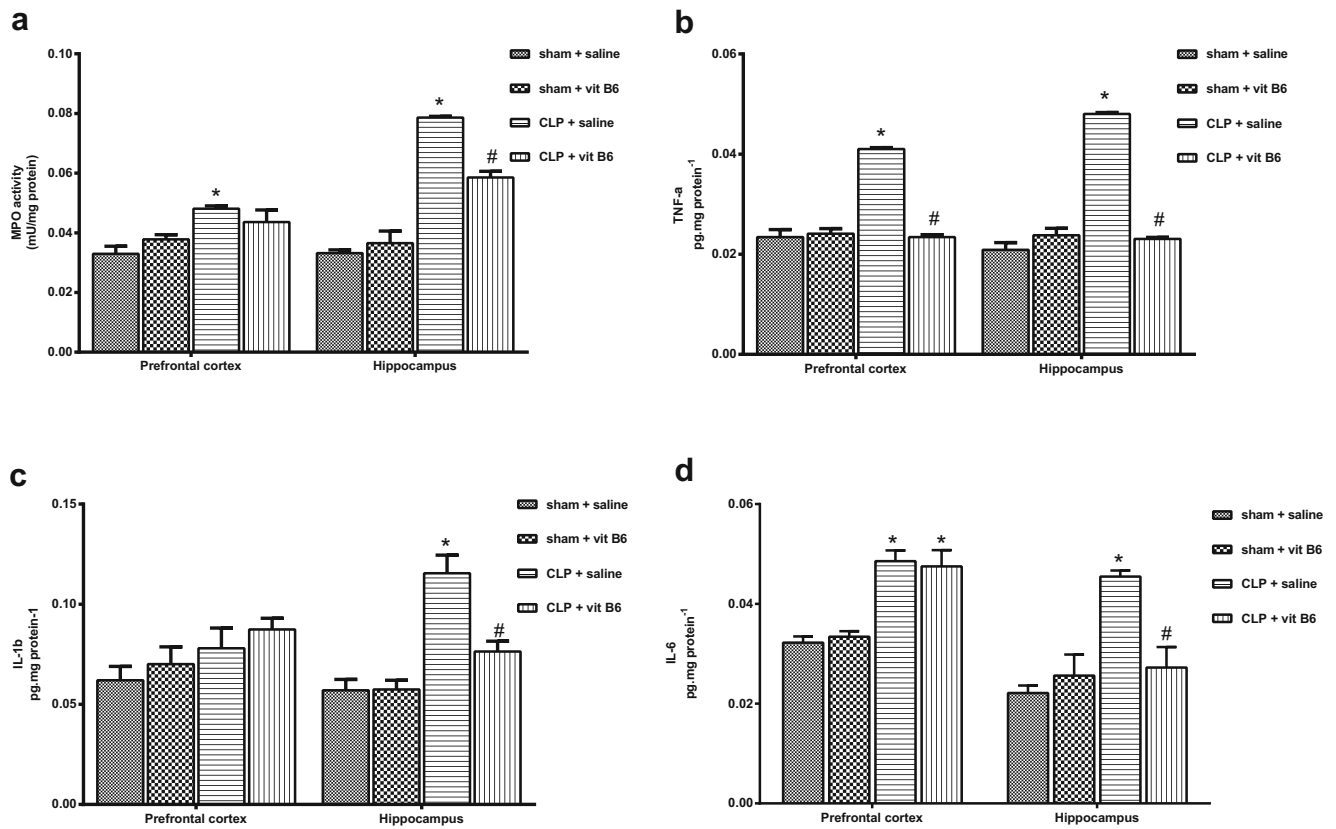


**Fig. 3** BBB permeability evaluation in the prefrontal cortex and hippocampus of rats subjected to polymicrobial sepsis and treated with vit B<sub>6</sub> (600 mg/kg). Data are expressed as mean ± standard deviation, analyzed by two-way ANOVA with post hoc Tukey. \**p* < 0.05 compared to sham + saline group and #*p* < 0.05 compared to CLP + saline group

significantly only in the hippocampus of CLP + saline animals, and vit B<sub>6</sub> treatment significantly reduced these levels. Sepsis elevated IL-6 levels (Fig. 4d) in the prefrontal cortex and hippocampus, compared to sham + saline rats, but vit B<sub>6</sub> prevented this increase only in the hippocampus.

### Vit B<sub>6</sub> Reduces Oxidative Stress and Changes in Brain Energy Metabolism after Sepsis

A significant increase in the nitrite/nitrate, an indicator of nitric oxide (NO) degradation, was visualized only in prefrontal cortex of animals submitted to sepsis (Fig. 5) and it was reversed by vit B<sub>6</sub> treatment. Lipid peroxidation was enhanced in both brain structures (Fig. 6a), while higher levels of protein carbonylation (Fig. 6b) were found only in the hippocampus of CLP + saline animals, compared to sham + saline rats. Septic animals receiving vit B<sub>6</sub>, on the contrary, were

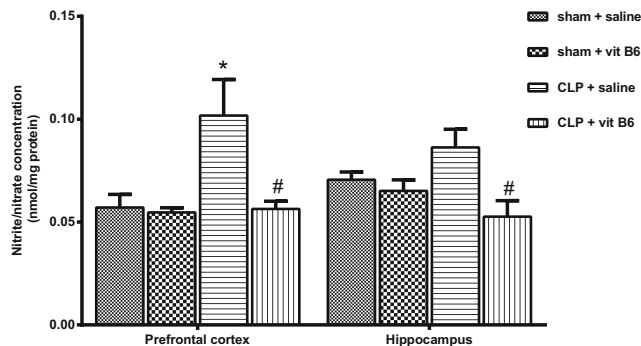


**Fig. 4** Myeloperoxidase activity (a) and levels of proinflammatory cytokines TNF- $\alpha$  (b), IL-1 $\beta$  (c), and IL-6 (d) in the prefrontal cortex and hippocampus of rats subjected to polymicrobial sepsis and treated with vit B<sub>6</sub> (600 mg/kg). Data are expressed as mean  $\pm$  standard

deviation, analyzed by two-way ANOVA with post hoc Tukey. \* $p < 0.05$  compared to sham + saline group and # $p < 0.05$  compared to CLP + saline group

prevented from lipid and protein oxidative damage caused by sepsis in these brain structures.

Panels c and d of Fig. 6, respectively, represent the enzymatic activities of SOD and CAT. Both enzymes had decreased activity only in the hippocampus of CLP + saline animals, compared to sham + saline rats. The administration of vit B<sub>6</sub> was effective in preserving SOD activity in this brain structure, but did not positively affect CAT activity.



**Fig. 5** Nitrite/nitrate concentration in the prefrontal cortex and hippocampus of rats subjected to polymicrobial sepsis and treated with vit B<sub>6</sub> (600 mg/kg). Data are expressed as mean  $\pm$  standard deviation, analyzed by two-way ANOVA with post hoc Tukey. \* $p < 0.05$  compared to sham + saline group and # $p < 0.05$  compared to CLP + saline group

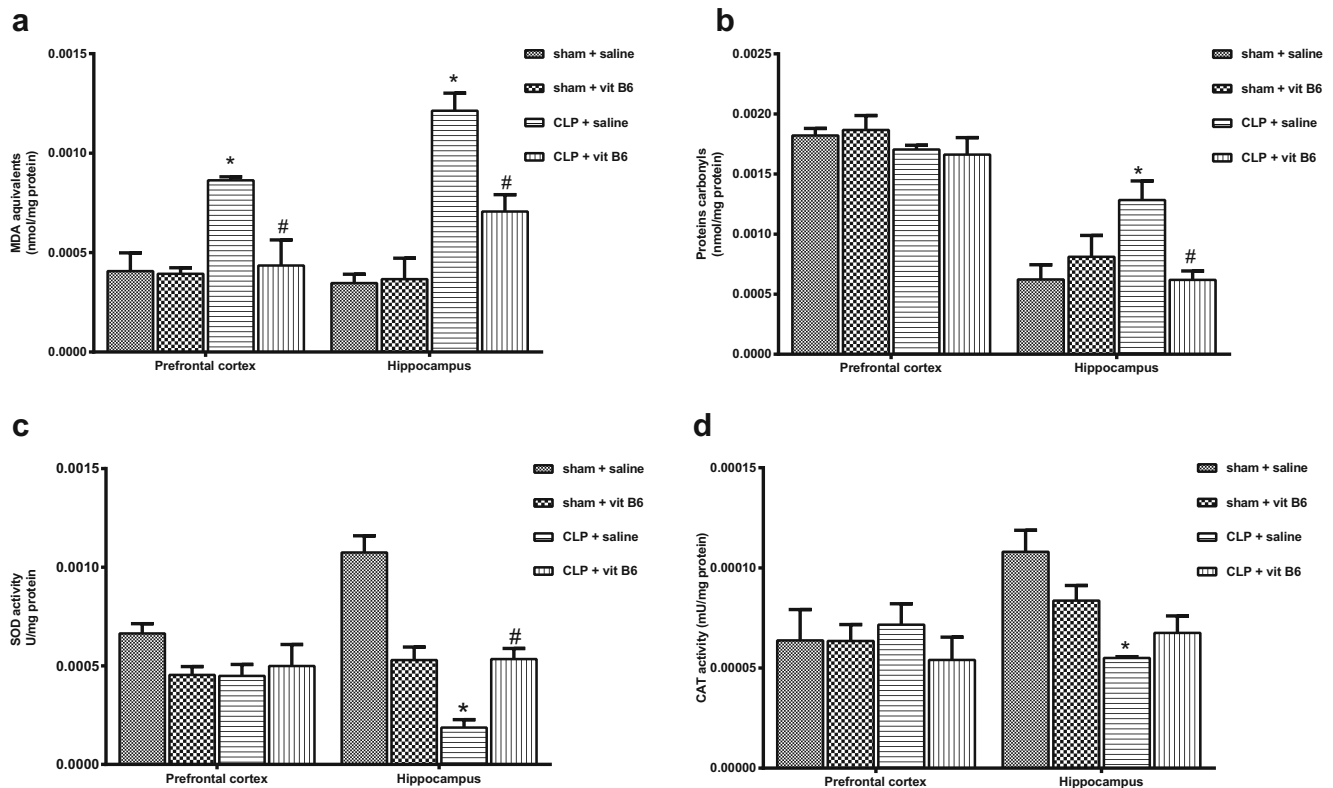
Regarding the activity of mitochondrial respiratory chain complex, a statistically significant increase was observed in the complex I activity (Fig. 7a) in the prefrontal cortex and hippocampus of CLP + saline rats. Vit B<sub>6</sub> reduced complex I activity only in the prefrontal cortex. The activity of complex II-III (Fig. 7b) and IV (Fig. 7c) was not altered in any group.

#### Vit B<sub>6</sub> Treatment Does Not Improve Mortality after Sepsis

Figure 8 shows the survival analysis. Animals submitted to sham + saline and sham + vit B<sub>6</sub> exhibited 100 and 93% survival rates, respectively, during the 10-day follow-up. Animals from CLP + saline group, on the other hand, presented a lower survival rate (31%), while 47% of CLP + vit B<sub>6</sub> animals survived at 10 days after sepsis induction, but there was no statistically significant difference among both septic groups.

#### Vit B<sub>6</sub> Treatment Improves Long-Term Cognitive Impairment in Sepsis Survivor Animals

Behavioral analyses demonstrate that CLP + saline animals presented deficits in motor performance and in non-associative memory, which can be observed in the crossing



**Fig. 6** Lipid peroxidation (a), protein carbonylation (b), SOD (c), and CAT (d) activities in the prefrontal cortex and hippocampus of rats subjected to polymicrobial sepsis and treated with vit B<sub>6</sub> (600 mg/kg).

Data are expressed as mean  $\pm$  standard deviation, analyzed by two-way ANOVA with post hoc Tukey. \* $p < 0.05$  compared to sham + saline group and # $p < 0.05$  compared to CLP + saline group

(Fig. 9a), rearing (Fig. 9b), and object recognition (Fig. 9c) measures, respectively. Conversely, the administration of vit B<sub>6</sub> significantly improved learning and memory deficits in septic rats in both behavioral tests (Fig. 9a–c).

## Discussion

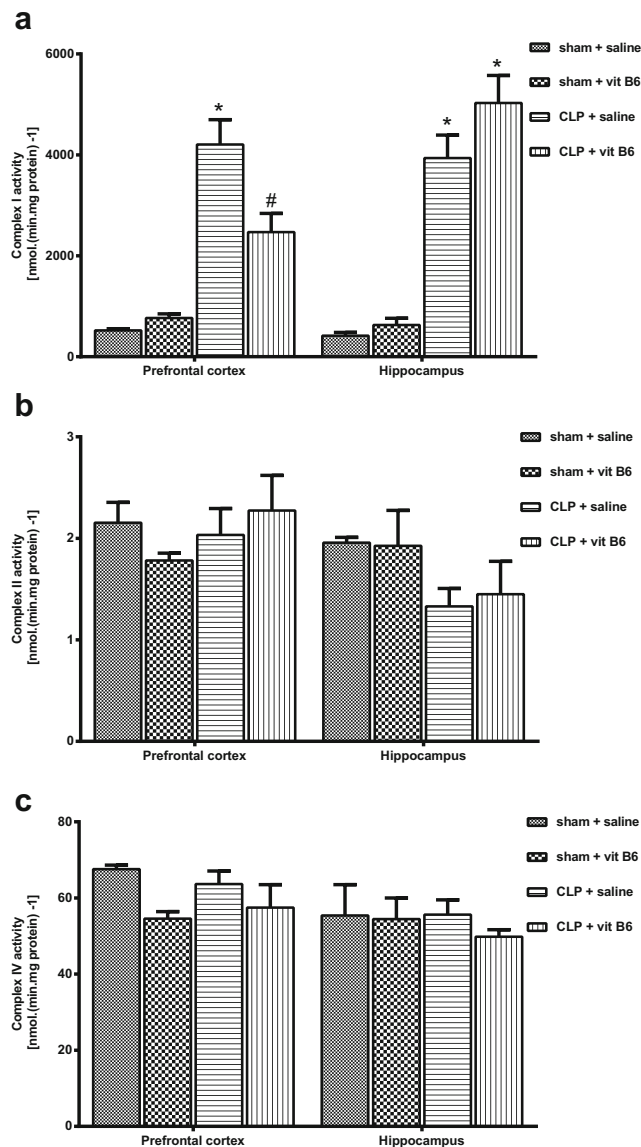
In this study, we report the acute and long-term neuroprotective effects of vit B<sub>6</sub> in a well-established model of experimental sepsis. The acute treatment with vit B<sub>6</sub> improved parameters related to brain dysfunction in sepsis, such as neuroinflammation, oxidative stress, BBB permeability, mitochondrial dysfunction, and long-term cognitive impairment. Additionally, here we present the kynurenine pathway activation in brain structures of septic rats and its modulation by vit B<sub>6</sub> treatment.

Our results suggest that, in CLP model, treatment with vit B<sub>6</sub> led to a pronounced increase in hippocampal and cortical kynurenine concentrations and a more discrete increase in tryptophan concentrations. This accumulation of kynurenine, unaccompanied by the proportional increase in concentrations of its precursor, indicates the reduction of kynurenine metabolism in response to treatment with B<sub>6</sub>. Kynurenine can be converted to quinurenic acid by the enzyme kynurenine

aminotransferase II (KAT-II); in anthranilic acid by the enzyme kynureninase (KYNU), or it can be hydroxylated by the enzyme kynurenine monooxygenase (KMO). Both the transformation of kynurenine by KYNU and KMO directs the route to its neurotoxic branch (3-hydroxyquinurenine, 3-hydroxyanthranilate, quinolinic acid). KAT-II and KYNU depend on B<sub>6</sub> as a cofactor [39], while KMO appears to be inhibited by this vitamin [40]. Importantly, as vit B<sub>6</sub> may regulate these key enzymes, vit B<sub>6</sub> treatment in this study may have influenced two major steps to reduce brain damage after sepsis and regulate KYN pathways: (1) inhibition of KMO activity as an enzymatic antagonist; and (2) enhancement of KAT-II activity as a crucial enzymatic cofactor. Both actions may lead to increased KYN levels and its direction to generate nontoxic metabolites, as we observed positive results regarding cognitive functions in CLP + vit B<sub>6</sub> animals.

It is well known that the systemic inflammatory response has a major effect on the development of SAE and long-term cognitive impairment [41, 42]. The metabolic fate of TRP, an essential amino acid, is to be converted into a variety of neuroactive substances, including the well-known neurotransmitters serotonin and melatonin. However, during inflammatory conditions, TRP is metabolized by the extrahepatic enzyme IDO to generate the toxic metabolite kynurenine [43, 44]. This is





**Fig. 7** Activity of complex I (a), complex II-III and complex IV (b) in the prefrontal cortex and hippocampus of rats subjected to polymicrobial sepsis and treated with vit B<sub>6</sub> (600 mg/kg). Data are expressed as mean ± standard deviation, analyzed by two-way ANOVA with post hoc Tukey. \* $p < 0.05$  compared to sham + saline group and # $p < 0.05$  compared to CLP + saline group

followed by the synthesis of neurotoxic molecules, such as anthranilic acid, xanthurenic acid, and quinolinic acid, among others, which act as *N*-methyl-D-aspartate receptor (NMDA) receptor agonists [40]. Sustained activation of NMDA receptors leads to excitotoxicity, a toxic event characterized by continual influx of calcium into the cell that activates the formation of reactive species, culminating in mitochondrial dysfunction.

In metabolomics analysis, KYN has been identified as a predictor of mortality in critically ill [10] and in trauma patients, acting as a clinical outcome indicator for sepsis. Also, increased plasma levels of KYN were associated

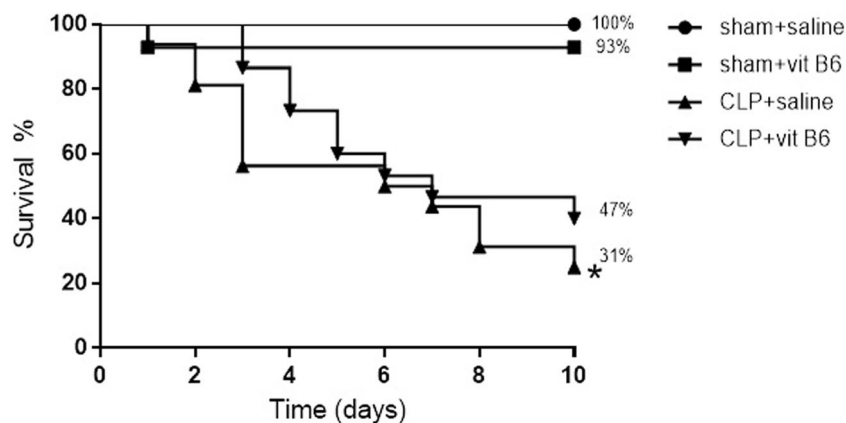
with the occurrence of acute brain dysfunction in critically ill patients [45]

With regard to IDO activation, evidences suggest that, in addition to several inflammatory mediators, lipopolysaccharide (LPS) can activate IDO in the brain [46, 47], mainly by stimulation of microglial cells [48]. In patients with sepsis, an increase in the activity of plasmatic IDO is directly proportional to sepsis severity, but this scenario can also be observed in sepsis survivors [11]. Recently, a sepsis study using 1-methyl-L-tryptophan, an IDO inhibitor, showed preservation of TRP levels and a decreased conversion to KYN [49].

Additionally, low concentrations of plasma vit B<sub>6</sub> are related to increased levels of proinflammatory cytokines [42], and vit B<sub>6</sub> supplementation in patients with rheumatoid arthritis was effective in reducing the levels of these cytokines [43]. Immune components, like cytokines, can actively cross the BBB during systemic inflammation [50]. Experimental studies with sepsis models implicate cytokines and reactive species in the increase of BBB permeability and the subsequent edema [49, 51]. The mechanisms that lead to BBB dysfunction are not fully understood, but include the activation, by cytokines and reactive species, of proteases called matrix metalloproteinases (MMP) subtypes 2, 8, and 9, which degrade key proteins responsible to maintain the BBB functionality [51]. We previously demonstrated that sepsis causes an increased BBB permeability in 24 h after CLP surgery [52]. Another study with using CLP model showed rolling processes and neutrophil adhesion in brain microvasculature that were associated with greater cell damage [52]. Also, a study involving patients with systemic inflammation indicated that plasma vit B<sub>6</sub> levels were inversely associated with the neutrophil counting in the systemic circulation [19]. Here we verified that vit B<sub>6</sub> reduced neutrophil infiltration and, consequently, prevented the damage to BBB permeability in the prefrontal cortex and hippocampus 24 h after sepsis induction.

The increased BBB permeability after sepsis allows not only activated neutrophils but also other circulating molecules to easily permeate the brain, like LPS, cytokines, and reactive species. When glial cells recognize these molecules, the result is an activation of different intracellular pathways with a consequent proinflammatory response [53]. Thus, we found that animals subjected to sepsis had elevated TNF- $\alpha$  and IL-6 levels in the prefrontal cortex and hippocampus and IL-1 $\beta$  only in the hippocampus. Previous studies performed in mouse macrophage RAW264.7 cells indicate that vit B<sub>6</sub> displays an important ability to block the effect of LPS in activating the transcription nuclear factor-kappa B (NF- $\kappa$ B) and the NLR family pyrin domain containing 3 (NLRP3) inflammasome, culminating in a suppression of IL-1 $\beta$  production and the inflammatory response [54, 55]. In this study, vit B<sub>6</sub> administered immediately after sepsis induction was effective in reducing

**Fig. 8** Kaplan-Meier curve for survival analysis of rats subjected to polymicrobial sepsis and treated with vit B<sub>6</sub> (600 mg/kg) following 10 days. Data analyzed by long-rank. \**p* < 0.05 compared to sham + saline group



TNF- $\alpha$  levels in both structures and IL-6 and IL-1 $\beta$  in the hippocampus.

It is noteworthy that inflammatory infiltrate is dominated by neutrophils, which have a considerable capacity to generate ROS and RNS [56]. In this sense, NO, although it plays a physiological role when presented in low concentrations, if available in concentrations beyond a critical level, it becomes enormously cytotoxic. This cytotoxicity occurs mainly due to the reaction with superoxide anion, which in turn forms extremely toxic peroxynitrite, or it can cause cell injury by interacting with proteins, metals, iron-sulfur-containing or heme-containing compounds [57]. Peroxynitrite formation has been proposed as a terminal event contributing to cell death and tissue injury in human sepsis [58], especially in the brain [58–62].

We found that vit B<sub>6</sub> reduced the levels of nitrite/nitrate, as a NO marker, in the prefrontal cortex and hippocampus after CLP surgery. A previous study demonstrated that vit B<sub>6</sub> can suppress inducible nitric oxide synthase (iNOS) expression induced by LPS, i.e., reduced levels of vit B<sub>6</sub> can increase the expression of iNOS, generating higher concentrations of NO [63].

Following nitrite/nitrate decrease, vit B<sub>6</sub> prevented lipoperoxidation in both structures, while prevented oxidative damage to proteins in the hippocampus. Vit B<sub>6</sub> exhibits an important antioxidant role [64] and its chemical structure is closely related to the ability of eliminating oxidants compounds [65]. Decreased levels of vit B<sub>6</sub> are related to elevated lipid peroxidation in plasma [66]. In vitro, vit B<sub>6</sub> prevented the formation of O<sub>2</sub><sup>-</sup> and lipid peroxidation [65], and in patients with Alzheimer's disease vit B<sub>6</sub> supplementation significantly decreased the levels of protein carbonyls [67]. In addition, vit B<sub>6</sub> is a cofactor of KYN pathway, it promotes the balance between kynurenic acid, quinolinic acid, and xanthurenic acid [68]. Quinolinic acid and xanthurenic acid are metabolites with direct oxidizing ability and may act as agonists of NMDA receptors, leading to excitotoxic events through the

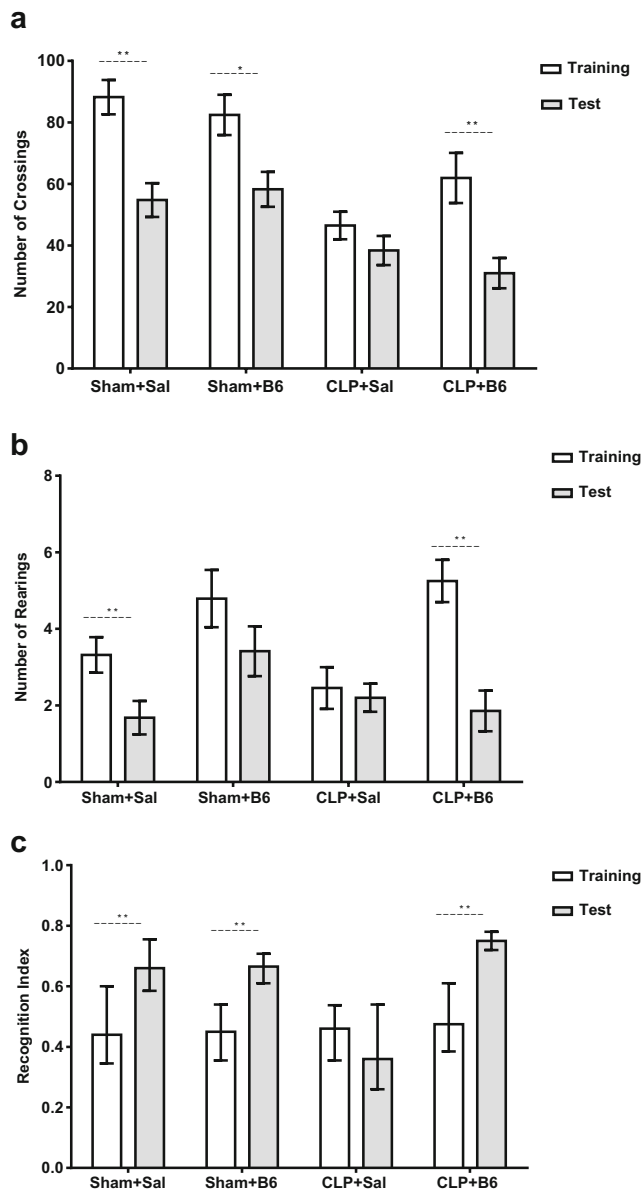
formation of ROS and RNS. Kynurenic acid, on the other hand, acts as an antagonist of NMDA receptors, besides having antioxidant effects [69].

The vulnerability of brain to oxidative stress seems to occur not only because this organ utilizes about one fifth of the total oxygen demand of the body, but also due to a smaller pool of antioxidant enzymes, compared to other organs [58]. In the present study, we found that vit B<sub>6</sub> prevented the decrease in SOD activity in hippocampus, contributing to the reduction of oxidative damage generated after sepsis. Higher levels of vit B<sub>6</sub> are positively associated with SOD activity in surgical patients [15]. Thus, adequate levels of vit B<sub>6</sub> proved to be an important factor to balance the concentration of pro- and antioxidant molecules.

On the other hand, vit B<sub>6</sub> had no effect in CAT activity. Among different functions of vit B<sub>6</sub>, it has an intrinsic relationship with the synthesis of glutathione peroxidase (GPx); thereby, it is possible that this vitamin positively exerted some effect on the GPx availability, which may have contributed to the protective effect against oxidative stress [70, 71].

A major source of ROS production during sepsis is the interaction between the mitochondrial electron transport chain enzymes, leading to changes in cell energy production and enhancing oxidative stress [72]. Here it was identified a significant elevation in the complex I activity in septic animals, compared to control animals, and it was prevented by vit B<sub>6</sub> in the prefrontal cortex. As vit B<sub>6</sub> acts like a cofactor in the catabolism of amino acids during the urea cycle, it can increase the availability of precursors to generate ATP, thus regulating cellular energy metabolism [73]. In this research, vit B<sub>6</sub> may have reduced the oxidative damage to proteins of mitochondrial respiratory chain by regulating the activity of complex I.

Despite the positive results related to vit B<sub>6</sub> treatment on neuroinflammation and oxidative stress, this treatment was not associated with higher survivor rates. Gao and colleagues also did not observe an improvement in survival after



**Fig. 9** Number of crossings (a), rearings (b), and object recognition index (c) of rats subjected to polymicrobial sepsis and treated with vit B<sub>6</sub> (60.0 mg/kg) 10 days after sepsis induction. Data presented as mean  $\pm$  standard deviation, analyzed by paired *t* test and two-way ANOVA with post hoc Tukey for crossing and rearings. Data presented as mean and interquartile range, analyzed by Mann-Whitney *U* tests. Comparisons between groups in the recognition index objects were performed using Wilcoxon test. \*\**p* < 0.01 and \**p* < 0.05 compared to the training session

administering an IDO inhibitor [74], although they have shown enhancements in memory and cognition as a result of decreased levels of KYN and its metabolites.

Since the control of neuroinflammation did not influence mortality in our model, we aimed at investigating if vit B<sub>6</sub> treatment could improve long-term brain dysfunction in sepsis survivors. Human studies show that sepsis leads to cognitive and structural damage in adults [75, 76] and causes cognitive

impairment and poor school performance in children who recovered from SAE [77]. We had previously demonstrated that controlling acute neuroinflammation was associated with improved long-term outcome in the CLP model of sepsis [40, 44, 65–67]. Here we demonstrated that vit B<sub>6</sub> also improves long-term cognitive dysfunction after sepsis recovery in rats.

Activation of KYN pathway can cause behavioral disorders, cognitive and memory impairment [78, 79]. In addition, KYN activation was found in the hippocampus and could explain neurocognitive alterations [80]. Furthermore, IDO activation can lead to decreased levels of serotonin, an important neurotransmitter involved in memory, and diminish the TRP availability [81].

After absorption, significant plasma levels of vit B<sub>6</sub> can last up to 20 days [82], but knowing that inflammatory processes lead to vit B<sub>6</sub> depletion, we cannot assume that animals presented a sufficient level of vit B<sub>6</sub> in the systemic circulatory system to have any long-term effect at the time of behavioral tests. However, it was demonstrated that vit B<sub>6</sub> exerts anti-inflammatory, antioxidant, and possibly a KYN pathway regulatory effect 24 h after sepsis induction. Thus, the long-term beneficial effects observed in this study may be due to the acute neuroprotection role exerted by vit B<sub>6</sub>. A recent study, using the same dose of vit B<sub>6</sub> and behavioral tests, showed improved cognition and memory 10 days after meningitis induction [21]. Furthermore, vit B<sub>6</sub> is a cofactor in the formation of serotonin pathway [83] and could improve memory and cognition. Importantly, the short-term neuroprotective effects of vit B<sub>6</sub> in our study were measured in the prefrontal cortex and hippocampus, which are brain structures related to memory and cognition [38].

## Conclusion

The administration of vitamin B<sub>6</sub> was effective in preserving the BBB integrity and, consequently, decreasing neuroinflammation and oxidative stress, which resulted in long-term memory and cognition improvement. Thus, in our experimental model, vit B<sub>6</sub> increased tryptophan and kynurenine levels in the prefrontal cortex and hippocampus and it may have contributed to protect these brain regions from the damage after sepsis.

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## Compliance with Ethical Standards

**Conflict of Interest** None of the authors or funding sources has conflict of interest.

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