



Immunopharmacology and inflammation

Cannabidiol reduces host immune response and prevents cognitive impairments in Wistar rats submitted to pneumococcal meningitis

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ABSTRACT

Pneumococcal meningitis is a life-threatening disease characterized by an acute infection affecting the pia matter, arachnoid and subarachnoid space. The intense inflammatory response is associated with a significant mortality rate and neurologic sequelae, such as, seizures, sensory-motor deficits and impairment of learning and memory. The aim of this study was to evaluate the effects of acute and extended administration of cannabidiol on pro-inflammatory cytokines and behavioral parameters in adult Wistar rats submitted to pneumococcal meningitis. Male Wistar rats underwent a cisterna magna tap and received either 10 μ l of sterile saline as a placebo or an equivalent volume of *S. pneumoniae* suspension. Rats subjected to meningitis were treated by intraperitoneal injection with cannabidiol (2.5, 5, or 10 mg/kg once or daily for 9 days after meningitis induction) or a placebo. Six hours after meningitis induction, the rats that received one dose were killed and the hippocampus and frontal cortex were obtained to assess cytokines/chemokine and brain-derived neurotrophic factor levels. On the 10th day, the rats were submitted to the inhibitory avoidance task. After the task, the animals were killed and samples from the hippocampus and frontal cortex were obtained. The extended administration of cannabidiol at different doses reduced the TNF- α level in frontal cortex. Prolonged treatment with cannabidiol, 10 mg/kg, prevented memory impairment in rats with pneumococcal meningitis. Although descriptive, our results demonstrate that cannabidiol has anti-inflammatory effects in pneumococcal meningitis and prevents cognitive sequel.

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1. Introduction

Pneumococcal meningitis is a complex and serious central nervous system (CNS) bacterial-induced inflammation, affecting the pia mater, arachnoid and subarachnoid space (Kim, 2008). *Streptococcus pneumoniae* meningitis is associated with a significant mortality rate and persisting neurologic sequelae, such as; seizures, sensory-motor deficits and impairment of learning and memory in up to 30% of surviving patients (Grimwood et al., 2000; Grandgirard et al., 2007; Hoogman et al., 2007). Histopathological human data

showed that the parenchymal damage was caused by an increase of the intracranial hypertension, interstitial edema and leukocyte infiltration (Nau et al., 1999).

The bacterial replication into subarachnoid space releases several compounds, such as, lipoteichoic acid, peptidoglycan and pneumolysin, which are recognized by Toll-like 2 and Toll-like 4 receptors, by antigen-presenting cells (Mook-Kanamori et al., 2011). Furthermore, meningeal and perivascular macrophages of CNS play a protective role during bacterial meningitis (Polfliet et al., 2001). They have been found to be capable of sensing pneumococci and produced pro-inflammatory cytokines (Zysk et al., 1997; Polfliet et al., 2001).

Tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) are the early response cytokine after pneumococcal meningitis (Täuber and Moser, 1999). For instance,

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patients with bacterial meningitis have increased TNF- α levels in cerebrospinal fluid (CSF) in the early course of the disease (Glimaker et al., 1993). The effects of TNF- α *in vivo* include fever, IL-1 β production by vascular endothelium cells and leukocyte activation (Saukkonen et al., 1990). This recruitment of polymorphonuclear leukocytes may induce damage not only to the bacteria but also to the brain (Mook-Kanamori et al., 2011).

In animal model of meningitis the TNF- α , IL-1 β , IL-6 and CINC-1 was produced in the first hours after induction (Barichello et al., 2010a). TNF- α administration into CSF, results in similar pathophysiological characteristic of bacterial meningitis (Rosenberg et al., 1995). Furthermore, intrathecal administration of anti-TNF- α antibody inhibited leukocytosis and brain edema in rabbit models of pneumococcal meningitis (Saukkonen et al., 1990). A central role of the host's inflammatory response in causing cerebral aggravation has been increasingly accepted (Sellner et al., 2010).

Thus, with an anti-inflammatory effect (Zuardi, 2008), cannabidiol (CBD) is the main non-psychotropic cannabinoid component derived from the *Cannabis sativa* plant (Izzo et al., 2009), it has antioxidant, neuroprotective properties and analgesic effects (Zuardi, 2008). Previous studies demonstrated that CBD reversed oxidative stress, cognitive impairment and mortality in a sepsis animal model (Cassol-Jr et al., 2010). It also decreased inflammation in a murine model of lung injury (Ribeiro et al., 2012) and reduced brain damage in hypoxic-ischemic model (Alvarez et al., 2008).

The aim of the present study was to evaluate the effects of acute and extended administration of CBD on the release of pro-inflammatory cytokines and behavioral parameters in adult Wistar rats submitted to pneumococcal meningitis.

2. Materials and methods

2.1. Infecting organism

S. pneumoniae (serotype 3) was cultured overnight in 10 ml of Todd Hewitt broth, diluted in fresh medium and grown to logarithmic phase. This culture was centrifuged for 10 min at (5000 \times g) and resuspended in sterile saline to the concentration of 5×10^9 cfu/ml. The size of the inoculum was confirmed by quantitative cultures (Grandgirard et al., 2007; Barichello et al., 2010a).

2.2. 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, Brazil, and followed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23) revised in 1996. 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) (Barichello et al., 2009). Rats underwent a cisterna magna tap with a 23-gauge needle. The animals received either 10 μ l of sterile saline as a placebo or an equivalent volume of *S. pneumoniae* suspension. At the time of inoculation, animals received fluid replacement and were subsequently returned to their cages (Irazuzta et al., 2002; Irazuzta et al., 2008). Meningitis was documented by a quantitative culture of 5 μ l of CSF obtained by puncture of the cisterna magna (Barichello et al., 2010a).

2.3. Drugs and treatment

CBD (THC-Pharm, Frankfurt, Germany, and STI-Pharm, Brentwood, UK) was suspended in polyoxyethylenesorbitan monooleate

(Tween 80) 2% saline. The solutions were prepared immediately before use and were protected from the light during the experimental session. For the treatment, CBD was administered *i.p.* immediately after meningitis induction at the doses of 2.5, 5 and 10 mg/kg (Zuardi et al., 2006): with either a single dose at day zero (for the animals in the acute treatment) or one dose daily for 9 days (for the animals in the extended treatment). All treatments were administered in a volume of 1 ml/kg of CBD plus vehicle. For this experiment we employed $n=5-6$ and $n=10$ for acute and extended treatments, respectively in each sub-group. The animals were divided into five sub-groups, as described below: sham; meningitis; meningitis+CBD 2.5 mg/kg; meningitis+CBD 5 mg/kg and meningitis+CBD 10 mg/kg.

2.4. Assessment of TNF- α , IL-1 β , IL-6 and CINC-1 concentrations

The brain structures, hippocampus, frontal cortex and CSF were immediately isolated on dry ice and stored at -80°C for analyses of the TNF- α , IL-1 β , IL-6, and cytokine-induced neutrophil chemoattractant (CINC-1) levels. Briefly, hippocampus and frontal cortex were homogenized in extraction solution containing aprotinin (100 mg of tissue per 1 ml). The CSF volume from each animal was adjusted to 100 μ l with extraction solution. The concentration of cytokines/chemokine was determined in hippocampus and frontal cortex using commercially available ELISA assays, following the instructions supplied by the manufacturer (DuoSet kits, R&D Systems; Minneapolis). The results are shown in pg/100 mg of tissues in frontal cortex and in hippocampus.

2.5. Assessment of BDNF

Brain-derived neurotrophic factor (BDNF) levels in hippocampus and frontal cortex were measured by anti-BDNF sandwich-ELISA, according to the manufacturer's instructions (Chemicon, USA). Briefly, rat hippocampus and frontal cortex were homogenized in phosphate buffer solution (PBS) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM (EGTA). Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in a sample diluent and the standard curve ranged from 7.8 to 500 pg/ml of BDNF. The plates were then washed four times with sample diluent and a monoclonal anti-BDNF rabbit antibody diluted 1:1000 in sample diluent was added to each well and incubated for 3 h at room temperature. After washing, a peroxidase conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After addition of streptavidin-enzyme, substrate and stop solution, the amount of BDNF was determined by absorbance in 450 nm and expressed as pg per mg wet tissue protein. The standard curve demonstrates a direct relationship between Optical Density (OD) and BDNF concentration. Total protein was measured by Lowry et al. (1951) method using bovine serum albumin as a standard, as previously described by Frey et al. (2006).

2.6. Step-down inhibitory avoidance apparatus and procedures

Ten days after pneumococcal meningitis the animals that received the extended treatment for 9 days with CBD were used for memory evaluation. The step-down inhibitory avoidance task was carried out in the apparatus with a $50 \times 25 \times 25$ cm acrylic box with the floor consisting of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7 cm-wide by 2.5 cm-high platform was placed on the floor of the box against the left wall (Quevedo et al., 1999; Roesler et al., 2003). In the training trial, the 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. A retention test trial was performed 24 h after training. The retention test trial was procedurally identical to training, except that no foot shock was performed. Immediately after the behavioral test, the rats were killed and the hippocampus and pre-frontal cortex were removed and stored at -80°C (Izquierdo et al., 1998).

2.7. Statistics

The results were shown by mean \pm S.E.M. of 5–6 and 10 animals for acute and extended treatments in each sub-group. Differences among groups were evaluated by using analysis of variance (ANOVA) followed by Tukey post-hoc test. Data for inhibitory avoidance are presented as the median (interquartile range) of retention test latencies. Differences between training and test session latencies within each group were determined using the Wilcoxon test. Comparisons among groups were performed using Mann–Whitney U test. P values <0.05 were considered statistically significant.

3. Results

3.1. Acute treatment

We evaluated BDNF, TNF- α , IL-1 β , IL-6 and CINC-1 levels at 6 h after pneumococcal meningitis induction in different groups; sham; meningitis; meningitis+CBD 2.5 mg/kg; meningitis+CBD 5 mg/kg and meningitis+CBD10 mg/kg.

Fig. 1A and B shows the TNF- α level in the hippocampus and frontal cortex. We verified an increased level of TNF- α in the hippocampus and frontal cortex, however the CBD treatment did not revert these findings.

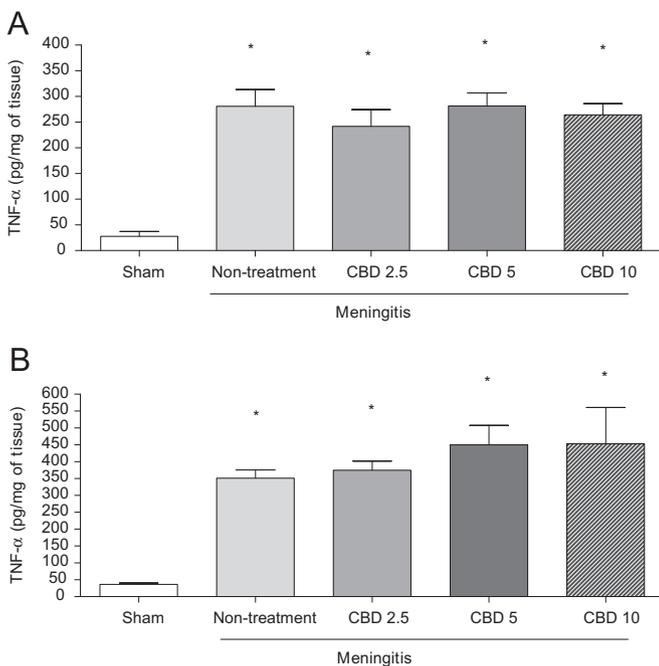


Fig. 1. Kinetics of TNF- α levels in hippocampus and frontal cortex 6 h after meningitis induction by *S. pneumoniae*. The concentrations of TNF- α levels in hippocampus (A) and frontal cortex (B) were obtained 6 h after meningitis induction. Levels of cytokines/chemokine were assessed by ELISA and results are shown as pg of cytokine/chemokine per 100 mg of tissue. Results show the mean \pm S.E.M. of 5–6 animals in each group. Symbols indicate statistically significant when compared with sham group $*P < 0.05$.

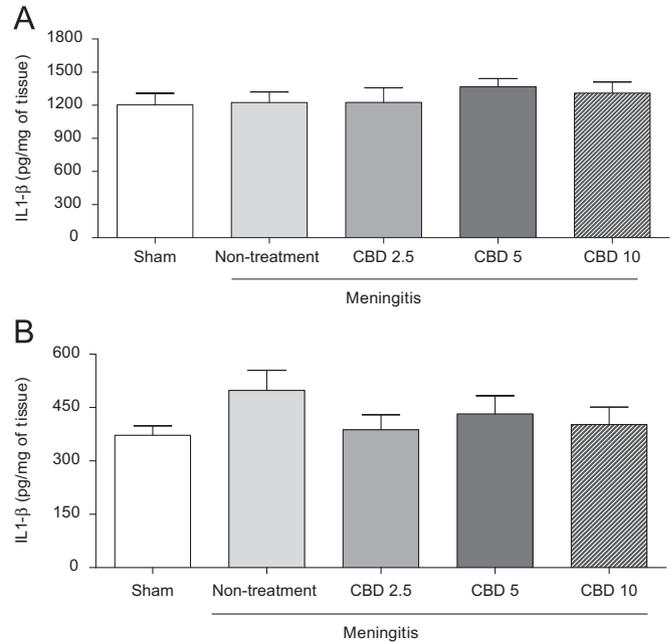


Fig. 2. Kinetics of IL-1 β levels in hippocampus and frontal cortex 6 h after meningitis induction by *S. pneumoniae*. The concentrations of IL-1 β levels in hippocampus (A) and frontal cortex (B) were obtained 6 h after meningitis induction. Levels of cytokines/chemokine were assessed by ELISA and results are shown as pg of cytokine/chemokine per 100 mg of tissue. Results show the mean \pm S.E.M. of 5–6 animals in each group. Symbols indicate statistically significant when compared with sham group $*P < 0.05$.

Figs. 2 and 3 show the IL-1 β and IL-6, respectively. The CBD treatment did not alter the IL-1 β and IL-6 levels in the hippocampus (Figs. 2A and 3A) and in frontal cortex (Figs. 2B and 3B).

Fig. 4A and B shows the CINC-1 levels in hippocampus and frontal cortex. In Fig. 4, the CINC-1 levels increased in both structures during the meningitis, however the CBD treatment did not revert these levels.

Finally, we analyzed the BDNF levels (Fig. 5A and B) in the hippocampus and frontal cortex. The meningitis process and the CBD treatment did not alter the BDNF levels in both structures.

3.2. Chronic treatment

In Fig. 6A and B we demonstrate the TNF- α level in the hippocampus and in frontal cortex. We can observe that meningitis did not change the TNF- α level in hippocampus 10 days after meningitis induction Fig. 6A. However, in the frontal cortex Fig. 6B the TNF- α levels increased after meningitis induction and CBD treatment with 2.5, 5 and 10 mg/kg decrease the high levels when compared with meningitis group without treatment ($P < 0.05$). Fig. 7A and B shows the BDNF levels in hippocampus and frontal cortex. The meningitis group without treatment decreases the BDNF level in frontal cortex ($P < 0.05$) Fig. 7B. However, Fig. 7B shows that there was no change in hippocampus. We can observe that CBD treatment in all doses increase the BDNF levels when compared with meningitis group without treatment ($P < 0.05$).

In the inhibitory avoidance test (Fig. 8), there was no difference between training session between groups. In the training and test sessions there were not statistical differences between meningitis, meningitis+CBD 2.5 mg/kg and meningitis+CBD 5 mg/kg, showing memory impairment in these groups. Nevertheless, the extended treatment with CBD+10 mg/kg prevented memory impairment when compared to meningitis group in the training session ($P < 0.05$).

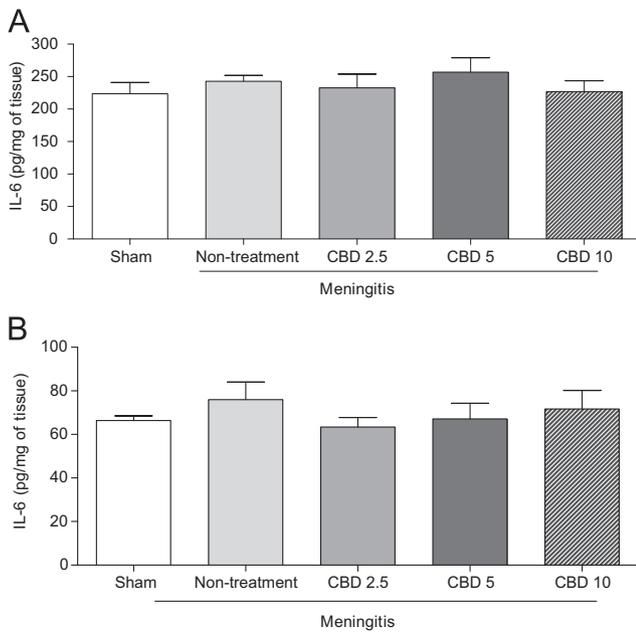


Fig. 3. Kinetics of IL-6 levels in hippocampus and frontal cortex 6 h after meningitis induction by *S. pneumoniae*. The concentrations of IL-6 levels in hippocampus (A) and frontal cortex (B) were obtained 6 h after meningitis induction. Levels of cytokines/chemokine were assessed by ELISA and results are shown as pg of cytokine/chemokine per 100 mg of tissue. Results show the mean \pm S.E.M. of 5–6 animals in each group. Symbols indicate statistically significant when compared with sham group $*P < 0.05$.

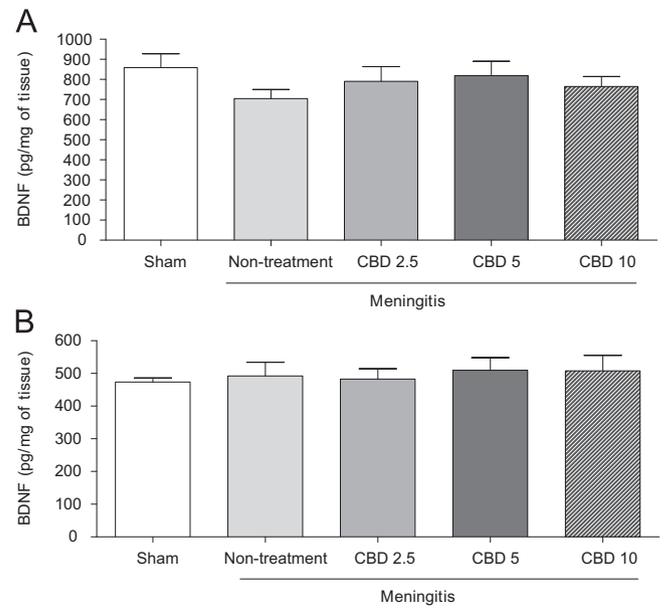


Fig. 5. Kinetics of BDNF levels in hippocampus and frontal cortex 6 h after meningitis induction by *S. pneumoniae*. The concentrations of BDNF levels in hippocampus (A) and frontal cortex (B) were obtained 6 h after meningitis induction. Levels of BDNF were assessed by ELISA and results are shown as pg of BDNF per 100 mg of tissue. Results show the mean \pm S.E.M. of 5–6 animals in each group. $***$ Symbols indicate statistically significant when compared with sham group $***P < 0.05$.

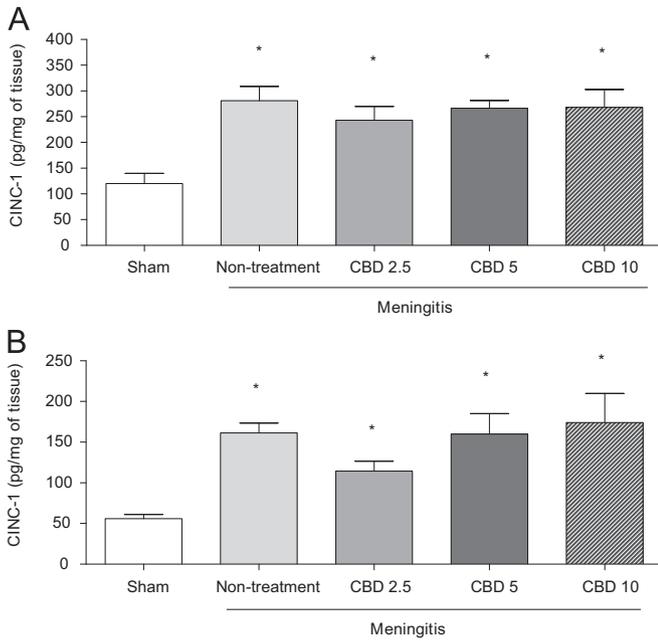


Fig. 4. Kinetics of CINC-1 levels in hippocampus and frontal cortex 6 h after meningitis induction by *S. pneumoniae*. The concentrations of CINC-1 levels in hippocampus (A) and frontal cortex (B) were obtained 6 h after meningitis induction. Levels of cytokines/chemokine were assessed by ELISA and results are shown as pg of cytokine/chemokine per 100 mg of tissue. Results show the mean \pm S.E.M. of 5–6 animals in each group. Symbols indicate statistically significant when compared with sham group $*P < 0.05$.

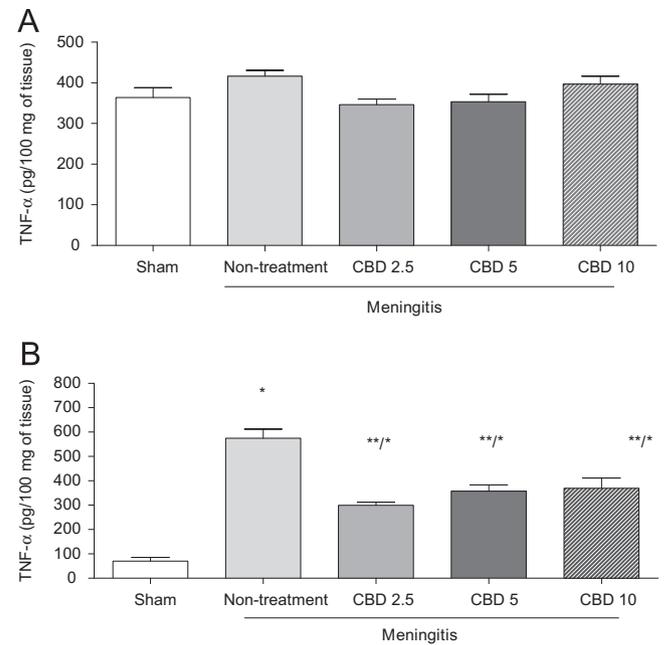


Fig. 6. Kinetics of TNF- α levels in hippocampus and frontal cortex 10 days after meningitis induction by *S. pneumoniae*. The concentrations of TNF- α levels in hippocampus (A) and frontal cortex (B) were obtained 10 days after meningitis induction. Levels of cytokines/chemokine were assessed by ELISA and results are shown as pg of BDNF per 100 mg of tissue. Results show the mean \pm S.E.M. of 5–6 animals in each group. $*$ Symbol indicates statistically significant when compared with sham group $*P < 0.05$. $**$ Symbols indicate statistically significant when compared with meningitis group without treatment $**P < 0.05$.

4. Discussion

Despite significant advances in pneumococcal meningitis treatment, it remains one of the most important worldwide infectious diseases and it is still associated with high mortality

and morbidity. In addition, many survivors present permanent neurological sequelae (Sellner et al., 2010; Mook-Kanamori et al., 2011). In the present study, we investigated the effects of acute and extended adjuvant treatment with CBD on pro-inflammatory cytokines and aversive memory.

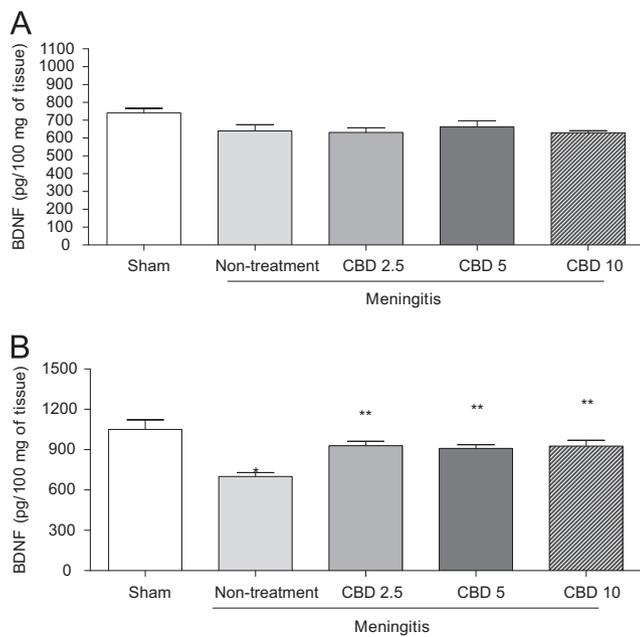


Fig. 7. Kinetics of BDNF levels in hippocampus and frontal cortex 10 days after meningitis induction by *S. pneumoniae*. The concentrations of BDNF levels in hippocampus (A) and frontal cortex (B) were obtained 10 days after meningitis induction. Levels of cytokines/chemokine were assessed by ELISA and results are shown as pg of BDNF per 100 mg of tissue. Results show the mean \pm S.E.M. of 5–6 animals in each group. ** Symbol indicates statistically significant when compared with sham group * $P < 0.05$.

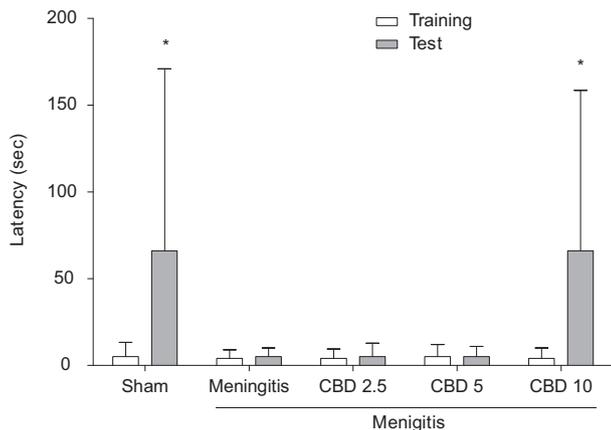


Fig. 8. Inhibitory avoidance test 10 days after induction of meningitis by *S. pneumoniae*. Data from the step-down inhibitory avoidance are presented as median and interquartile ranges, $n=10$ rats per group * $P < 0.05$ versus training. Comparisons among groups were performed using Mann–Whitney U tests. Within individual groups it was analyzed by Wilcoxon test.

Six hours after pneumococcal meningitis induction there was an increase in the levels of TNF- α and CINC-1 in hippocampus and frontal cortex. However, the administration of different doses of CBD did not reverse these levels in the hippocampus and frontal cortex. In previous studies, using the same rat model of meningitis, we verified the increase of TNF- α and CINC-1 levels in hippocampus and frontal cortex at 6 h after pneumococcal meningitis induction (Barichello et al., 2010a). Furthermore, in jugular plasma, the CINC-1 increased at 3 h staying elevated up to 6 h and TNF- α levels increased at 6 h. In arterial plasma we verified the increased of the CINC-1 at 6 h after induction, indicating that possibly CINC-1 and TNF- α are produced in the CNS (Barichello et al., 2012). TNF- α , IL-1 β and IL-6 are produced in the early response after pneumococcal meningitis induction

(Täuber and Moser, 1999). TNF- α and CINC-1 are also implicated in the infiltration of inflammatory cells into the brain parenchyma (Saukkonen et al., 1990; Katayama et al., 2009). Moreover, such indeterminate properties, prosperous actions and the exact site at which CBD could exert its neuroinflammatory and neuroprotective effects are still not completely elucidated. The recently discovered ability of different cannabinoids, including CBD, to display an extra-cannabinoid receptor binding activity has been highlighted by the observation that these compounds may go nuclear to exert their activity through the interaction with peroxisome proliferator-activated receptors (PPARs) (O'Sullivan et al., 2007). Furthermore, in the concentration 10 mg/kg of the CBD administered for 15 days reduced A β -induced neuroinflammation and promoted hippocampal neurogenesis through PPARs (Esposito et al., 2011). In a previous study we verified that pneumococcal meningitis also cause change of the mitochondrial respiratory chain (Barichello et al., 2010b) and oxidative stress (Barichello et al., 2010c). Furthermore, apoptosis in pneumococcal meningitis is correlated with production of the oxygen reactive species that causes mitochondrial dysfunction that leads to the release of the apoptosis-inducing factor into the cytosol (Mitchell et al., 2004).

Independent from classical CB1/2 receptors, the CBD (3 and 10 mg/kg) 2 h before of the hepatic ischemia reperfusion reduced the extent of liver inflammation, oxidative/nitrative stress and cell death (Mukhopadhyay et al., 2011). Hepatic ischemia reperfusion also leads to an early impairment of the activities of the enzymes of the mitochondrial respiratory chain, mitochondrial dysfunction, allowing more reactive oxygen species to leak out of the respiratory chain (Jaeschke et al., 2003; Moon et al., 2008). CBD likewise was reported to blunt tyrosine phosphorylation in cultured neurons by reducing phosphorylation of glycogen synthase kinase 3b (GSK3b), acting as a Wnt/b-catenin pathway rescuer, although alternative mechanisms may be implicated in inducing this effect (Esposito et al., 2006). GSK 3b is involved in toll-like receptor signaling and regulates the production of pro-inflammatory cytokines and septic shock (Woodgett and Ohashi, 2005). In another study, the chronic administration of CBD at different doses (2.5, 5, and 10 mg/kg, i.p., by seven days) also prevented memory impairment of rats 10 days after to sepsis by ceal ligation and perforation (Cassol-Jr et al., 2010).

Furthermore, CBD decreased TNF- α , IL-6 and chemokines (MCP-1 and MIP-2) in a murine model of acute lung injury (Ribeiro et al., 2012); synthetic cannabinoid inhibited TNF- α , IL-1 β and IL-6 expression induced by lipopolysaccharide in rat cerebellar granule cells (Chiba et al., 2011). CBD reduced blood brain-barrier alterations and prevented endothelial inflammatory response in arteriolar and venular vasodilation, induced by lipopolysaccharide (Ruiz-Valdepeñas et al., 2011). In a previous study we reported the increase of the TNF- α level in frontal cortex 10 days after meningitis induction (Barichello et al., 2010d). In this study, we observed in animals that received prolonged treatment with CBD decreased the TNF- α level in frontal cortex 10 days after meningitis induction. Furthermore, we evaluated the aversive memory with extended CBD treatment in different concentrations 2.5, 5 and 10 mg/kg, however, only the highest CBD dose prevented memory impairment. CBD in the same concentrations also prevented this impairment in rats submitted to sepsis (Cassol-Jr et al., 2010).

It has been well established that pneumococcal meningitis survivors present long-term cognitive impairment. We have already demonstrated that rats who survived pneumococcal meningitis presented learning and memory impairment 10 days after meningitis induction (Barichello et al., 2010e). *S. pneumoniae* replication within the subarachnoid space initiates a complex host immune response (Sellner et al., 2010), furthermore,

lipoteichoic acid and peptidoglycans are recognized by TLR-2 and pneumolysin by TLR-4 receptors presenting in antigen-presenting cells (Mook-Kanamori et al., 2011). When they are activated they induce the establishment of nuclear factor kappa B (NF- κ B) (Opitz et al., 2009). It is also a transcriptional activator of many genes involved in the pathogenesis of pneumococcal meningitis, such as, TNF- α , IL-1 β , nitric oxide synthase and intercellular adhesion molecules (Koedel et al., 2000; Kastenbauer et al., 2004). Some studies indicate that CBD exerts its anti-inflammatory effects through NF- κ B inhibition. For instance, CBD treatment inhibited the NF- κ B-dependent mRNA and TNF- α , in diabetic myocardial tissues (Rajesh et al., 2010). CBD also reduced IL-1 β , IL-6 and IFN- β and the activity of the NF- κ B pathway, a primary pathway regulating the expression of pro-inflammatory genes from lipopolysaccharide-activated in microbial cells (Kozela et al., 2010).

Nevertheless, new adjunctive therapies are needed to improve the prognosis of bacterial meningitis (Mook-Kanamori et al., 2011). Although descriptive, our results demonstrate that chronic treatment with CBD plays an anti-inflammatory role in pneumococcal meningitis. Furthermore, it prevents cognitive damage, possibly representing a new pharmacological approach towards pneumococcal meningitis. Future studies must be realized to understand the underlying mechanisms.

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