

# **The role of minocycline in cognitive impairment and dysfunction of the blood brain barrier in experimental pneumococcal meningitis**

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## Abstract

Bacterial meningitis is a life threatening infection associated with cognitive impairment in many survivors. The pathogen invades the CNS by penetrating through the luminal side of the cerebral endothelium, which is an integral part of the BBB. Microglia are the resident macrophages of the CNS which can trigger a host of immunological pathways. The inflammatory response from microglial activation can facilitate the elimination of invasive microorganisms; however, excessive or extended microglial activation can result in neuronal damage and eventually cell death. The inhibition of microglia using minocycline can be a relevant pharmacological tool to study the role of microglia in different CNS diseases. In this study, animals received either artificial cerebrospinal fluid or a *Streptococcus pneumoniae* suspension. The animals receive minocycline or saline immediately after induction. For the evaluation of the BBB integrity, the animals were killed at 12, 18 and 24 h after induction. For the behavioural tests, ten days after meningitis was induced, were subjected to open-field habituation and the step-down inhibitory task. In both cerebral structures the use of the minocycline prevented BBB disruption. In the behavioural tests the use of minocycline prevented habituation and aversive memory impairment in the meningitis/minocycline group when compared with meningitis/saline. Our results demonstrate that the minocycline was able to decrease long-term cognitive impairment and BBB dysfunction in rats survivors of meningitis representing a new pharmacological approach towards pneumococcal meningitis.

**Keywords:** Bacterial meningitis, minocycline, microglia, cognitive impairment, blood-brain barrier.

## **Introduction**

Bacterial meningitis is a life threatening disease with fatality rates of approximately 26% and it is supposed that brain dysfunction may be associated with long-term cognitive impairment in approximately 26% survivors [1,2]. Previous studies on cognitive outcomes after pneumococcal meningitis showed reduced psychomotor, slight mental slowness, impairment in attention and executive functions, and learning and memory deficiencies [2,1,3]. Replication of microorganism within the subarachnoid space occurs at the same time with the release of their compounds, such as peptidoglycan bacteria within the subarachnoid space occurs at the same time with the release of their compounds, such as peptidoglycan, lipoteichoic acid, flagellin, lipopolysaccharide (LPS), DNA and cell wall fragment. These compounds are known as pathogen-associated molecular patterns (PAMPs). These PAMPs are recognised by pattern-recognition receptors (PRRs), which are the pivotal components of the innate immune system [4,5] which stimulates the production of cytokines and other pro-inflammatory molecules in response to bacterial stimuli [6]. In response to inflammatory stimuli, leukocyte cells leave the blood and migrate into the site of infection [7]. These cells produce quantities of nitric oxide and superoxide anion, and this leads to lipid peroxidation, DNA single strand breaks, matrix metalloproteinase (MMP) activation, mitochondrial injury, blood-brain barrier (BBB) breakdown and brain impairment [8,9].

Microglia are known for playing a key role in mediating inflammatory processes associated with various diseases [10] and can be activated by many stimuli including PAMPs, DAMPs or pro-inflammatory mediators to produce cytokines, chemokines, and reactive oxygen and nitrogen species which are involved in eliminating the invading microorganism. Damage to the central nervous system (CNS) during bacterial

meningitis involves pathogenic mechanisms of bacteria and the innate immune host response [11,12]. Minocycline is a semisynthetic second generation tetracycline that have anti-inflammatory, antioxidant, anti-apoptotic, and neuroprotective effects on microglial cells in rodent models [13]. The inhibition of microglia using minocycline can be a relevant pharmacological tool to study the role of microglia in different CNS diseases [14].

The aim of this study was to investigate the effects of minocycline on integrity of BBB memory in an experimental model of pneumococcal meningitis.

## **2. Experimental procedures**

### *2.1. Infecting organisms*

*Streptococcus pneumoniae* serotype III was cultured overnight in 10 mL of Todd Hewitt Broth, Himedia® and then diluted in fresh medium and grown to logarithmic phase. The culture was centrifuged for 10 min at 5,000  $\times$  g and re-suspended in sterile saline to a concentration of  $5 \times 10^9$  colony-forming units (CFU). The size of the inoculum was confirmed by quantitative cultures [15].

### *2.2. Meningitis animal model*

Adult male 60-day-old Wistar rats (250-300 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 (029/2015-1), Brazil, and were 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 anaesthesia consisting of an intraperitoneal injection of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and

acepromazine (0.16 mg/kg) [16]. The animals received an intracisternal (i.c.) injection of 10  $\mu$ L of artificial cerebrospinal fluid (CSF) as a placebo or an equivalent volume of *S. pneumoniae* suspension. At the time of the inoculation, the animals received fluid replacement and were subsequently returned to their cages. Eighteen hours after the induction of meningitis, the infection was documented by a quantitative culture of 5  $\mu$ L of CSF by puncturing the cisterna magna [15].

### 2.3 Treatment

The animals receive minocycline (100  $\mu$ g/Kg icv) immediately after induction (dissolved in sterile saline, 0.5  $\mu$ L i.c., Sigma-Aldrich, Saint Louis, USA) or saline [17]. To evaluate the behavioural response, the animals were separated into four groups: control/saline, control/ minocycline, meningitis/saline, and meningitis/ minocycline (n = 10 animals per group; n = 80). Eighteen hours after induction, all animals received ceftriaxone (100 mg/kg body weight given s.c./7 days) [18]. To evaluate the BBB integrity, the animals were separated in the same groups.

### 2.4 BBB permeability to Evan's blue

We investigated the BBB integrity by Evan's blue dye extravasation (Smith and Hall, 1996) at 12, 18 and 24 h after pneumococcal meningitis induction (n = 5-6). One mL of Evan's blue at 1% was injected in all animals intraperitoneally 1 h before being killed. The anesthesia consisted of an intraperitoneal administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and acepromazine (0.16 mg/kg) [19]. The animal's chests were opened, and the brain was perfused with 200 mL of sterile saline through the left ventricle at a pressure of 100 mmHg until colorless perfusion fluid was found from the right atrium. The brains were weighed and stored in trichloroacetic solution at 50%

concentration. Ethanol (1:3) was used as diluent of extracted dye and its fluorescence was determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrophotometer (Hitachi 650-40, Tokyo, Japan). The tissue content Evan's blue was measured from a linear standard line derived from known quantities of the dye, and it was expressed per gram of tissue [20].

## *2.5 Behavioural tasks*

Ten days after inoculation, the animals were free from infection. All blood cultures that were performed during this period were negative. Finally, the animals were randomised and subjected to the open-field habituation and step-down inhibitory avoidance tasks.

### *2.5.1. Open field task*

Behaviour was assessed in an open-field apparatus to evaluate both locomotor and exploratory activity. The apparatus was a 40 × 60 cm open field surrounded by 50-cm-high dark grey walls and a front glass wall. Black lines divided the floor of the open field into nine rectangles. Each animal was gently placed in the centre of the open field and was left to explore the arena for 5 min (training session). The number of crossings (i.e., the number of times that the animal crossed the black lines, an assessment of locomotor activity) and rearing movements (i.e., the exploratory behaviour observed in rats subjected to a new environment) were measured. Immediately after this procedure, the animals were taken back to their home cage. Twenty-four hours later, they were subjected to a second open-field session (test session). In both sessions, the number of times the animal crossed the black lines or reared was counted during a 5-min period. The reduction in the number of crossings and rearings between the two sessions was

taken as a measure of the retention of memory. The behavioural test was performed by the same person (manual analyses) who was blind to the group treatment [21].

### *2.5.2. Step-down inhibitory avoidance task*

The apparatus and procedures have been described in previous reports [22,23]. Briefly, the training apparatus was a 50 x 25 x 25 cm acrylic box (Albarsch, Porto Alegre, Brazil), whose floor consisted of parallel calibre 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 administered. The retention test step-down latency (maximum, 180 s) was used as a measure of inhibitory avoidance retention [24,25]

## **3. Statistics**

Data were analysed for normality by the Shapiro–Wilk test and for homogeneity using the Levene test. When the data were normal and homogeneity of variance, parametric tests was used, not meeting this condition, non-parametric tests were used. The results of the BBB integrity are reported as the mean  $\pm$  SD of 5-6 animals in each group. Differences among groups were evaluated using analysis of variance (ANOVA) followed by the Student Newman–Keuls post-hoc test. Data from the inhibitory avoidance task are reported as median and interquartile ranges, the comparisons among

groups were performed using a Mann–Whitney U-test. The within-individual group comparisons were made by Wilcoxon's tests. Data from the habituation to open field task are reported as the mean  $\pm$  SD and comparisons among groups were analysed by paired Student's *t*-tests and analysis of variance with post-hoc Tukey's tests. P values  $*p < 0.05$  were considered statistically significant. All analyses were performed using the Statistical Package for the Social Science (SPSS) software version 20.0.

#### 4. Results

In figure 1, we evaluated BBB integrity in the hippocampus (A) and cortex (B) at 12, 18 and 24 h after pneumococcal meningitis induction in animals administered adjuvant treatment minocycline. In both structures, hippocampus and cortex, the use of the minocycline prevented BBB disruption ( $p < 0.05$ ).

Figures 2 and 3 illustrate the effects of minocycline on the memory of adult Wistar rats subjected to *S. pneumoniae*-induced meningitis. Meningitis group rats subjected to the open-field habituation task showed no difference between their training and test sessions, demonstrating memory impairment ( $p > 0.05$ ). However, in the control, control/ minocycline and meningitis/ minocycline groups, there were differences in the number of crossings and rearings between the training and test sessions, demonstrating memory habituation ( $p < 0.05$ ; figure 2A and 2B).

In the step-down inhibitory avoidance task, there was a difference between the training and test sessions in the control, control/minocycline and meningitis/minocycline groups, demonstrating aversive short- and long-term memory in these groups ( $p < 0.05$ ). In the meningitis group, there was no difference between the training and test sessions, demonstrating impairment of short- and long-term aversive



memory in these mice ( $p > 0.05$ ; figure 3).

## 5. Discussion

In the present study, we demonstrated the influence minocycline on BBB integrity and on learning and memory in an animal model of pneumococcal meningitis.

Microglia cells maintain cellular, synaptic, and myelin homeostasis during development, normal function of the CNS and in response to CNS injury. Thus, microglia have been shown to mediate a myriad of aspects of neuroinflammation, including recognition of pathogens bound to MHC for activation of T lymphocytes, phagocytosis, cytotoxicity through production of cytokines and secretion of glutamate, aspartate, reactive oxygen and nitrogen species [26].

In previous studies, we verified BBB disruption at 12, 18 and 24 h after pneumococcal meningitis induction in the hippocampus and cortex [27]. In this study, we observed the breakdown of the BBB in the hippocampus and cortex at 12, 18 and 24 h after induction and treatment with minocycline prevented this dysfunction. The immune response, the production of cytokines, and leukocyte migration are first line of defense against to bacterial infection [28]. Leukocytes produce nitric oxide, superoxide anion radicals and hydrogen peroxide that can lead to the formation of peroxynitrite [29], which is a strong oxidant that exerts cytotoxic effects on endothelial cells [30], increases the BBB permeability [31], and provokes lipid peroxidation, mitochondrial damage and matrix metalloproteinases activation [29]. The decrease of microglia activation by minocycline was able to prevent the disruption of the BBB in the hippocampus and cortex of Wistar rats after induction meningitis. Michels e co-works, demonstrated that use of minocycline prevented breakdown of the BBB in rats

submitted to experimental model of sepsis [32].

It has been well established that bacterial 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 pneumococcal meningitis induction [33]. The present study demonstrated that adjuvant treatment with minocycline prevented short- and long-term aversive memory impairments, as well as open-field habituation memory. The use of minocycline prevents long-term memory impairment and hippocampal inflammation in rats that are neonatally infected with *Escherichia coli* [34]. Lam and colleagues (2013) demonstrated that chronic administration of minocycline attenuated neuroinflammation and prevented injury memory impairment after traumatic brain injury [35].

Despite evidence that microglia activation is associated with brain injury, its role in the long-term cognitive impairment is not clear. Our results demonstrate that the inhibition of the microglia by minocycline was able to decrease long-term cognitive impairment and BBB dysfunction in rats survivors of bacterial meningitis possibly representing a new pharmacological approach towards pneumococcal meningitis. Future studies must be realized to understand the underlying mechanisms.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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## Legends to figures

**Fig. 1 Evaluated the integrity of BBB using Evan's blue dye extravasation in hippocampus (A) and cortex (B) at 12, 18 and 24 h after *S. pneumoniae* meningitis induction.** Differences among groups were evaluated using analysis of variance (ANOVA) followed by the Student Newman–Keuls post-hoc test. \* $p < 0.05$  indicates statistically significant differences between training and test sessions. #  $p < 0.05$  indicates statistical significance compared with the meningitis/saline group.

**Fig. 2 Effects of minocycline on the open-field habituation task by adult Wistar rats 10 days after the induction of pneumococcal meningitis.** The numbers of crossing and rearing movements are reported as the mean  $\pm$  SEM and were analysed by paired Student's *t*-tests and analysis of variance with post-hoc Tukey's tests (10 animals per group). \* $p < 0.05$  indicates statistical significance compared with training.

**Fig. 3 Effects of minocycline on the step-down inhibitory avoidance task in adult Wistar rats 10 days after the induction of pneumococcal meningitis.** Data are reported as median and interquartile ranges, and comparisons among groups were performed using Mann-Whitney U tests. The intra-group variations were analysed by Wilcoxon's tests. \* $p < 0.05$  indicates statistically significant differences between training and test sessions. &  $p < 0.05$  indicates statistical significance compared with the meningitis/saline group.