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Research Report

Brain–blood barrier breakdown and pro-inflammatory mediators in neonate rats submitted meningitis by *Streptococcus pneumoniae*

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ABSTRACT

Neonatal meningitis is an illness characterized by inflammation of the meninges and occurring within the birth and the first 28 days of life. Invasive infection by *Streptococcus pneumoniae*, meningitis and sepsis, in neonate is associated with prolonged rupture of membranes; maternal colonization/illness, prematurity, high mortality and 50% of cases have some form of disability. For this purpose, we measured brain levels of TNF-α, IL-1β, IL-6, IL-10, CINC-1, oxidative damage, enzymatic defense activity and the blood–brain barrier (BBB) integrity in neonatal Wistar rats submitted to pneumococcal meningitis. The cytokines increased prior to the BBB breakdown and this breakdown occurred in the hippocampus at 18 h and in the cortex at 12 h after pneumococcal meningitis induction. The time-dependent association between the complex interactions among cytokines, chemokine may be responsible for the BBB breakdown and neonatal pneumococcal severity.

Abbreviations: BBB, blood–brain barrier; CAT, catalase; CINC-1, cytokine-induced neutrophil chemoattractant-1; CSF, cerebral spinal fluid; CNS, central nervous system; CFU, colony-forming units; DNPH, dinitrophenylhidrazine; IL, interleukin; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NF-kB, nuclear factor kappa B; PMN, polymorphonuclear; ROS, reactive oxygen species; SOD, superoxide dismutase; S. *agalactiae*, *Streptococcus agalactiae*; S. *pneumonia*, *Streptococcus pneumoniae*; TNF-α, tumor necrosis factor-alpha; L. *monocytogenes*, *Listeria monocytogenes*; TBARS, thiobarbituric acid reactive species

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1. Introduction

Neonatal meningitis is an illness characterized by inflammation of the meninges and occurring within the first 28 days of life. Being that, this illness remains an important cause of mortality and morbidity in newborns (de Louvois et al., 2004; Kim, 2010). The microorganism causing neonatal meningitis varies from each country; however, a relatively small number of the pathogens, such as, *Streptococcus agalactiae*, *Escherichia coli*, *Listeria monocytogenes*, *Haemophilus influenzae* type b, *Neisseria meningitis* and *Streptococcus pneumoniae* (Kim, 2010). *S. agalactiae* is implicated in up to 50% of cases; in developing countries the Gram negative bacilli accounts for 30–40% (Dawson et al., 1999); being unusual with the presence of *S. pneumoniae* 6% and *L. monocytogenes* 5% (Heath and Okike, 2010). However, pneumococcal meningitis is the most severe bacterial meningitis. Previous reports suggest that invasive infection by *S. pneumoniae*, meningitis and sepsis; in neonatal is associated with prolonged rupture of membranes, maternal colonization/illness, prematurity and high mortality (50%) (Hoffman et al., 2003). The mortality of untreated patients approaches 100% (Kim, 2010) and 50% of treated cases have some form of disability (Heath and Okike, 2010). *S. pneumoniae* crosses the blood–brain barrier (BBB) and colonizes the subarachnoid space. Polymorphonuclear leukocytes (PMN) are the first defense from bacterial infection, consequently, high amounts of myeloperoxidase (MPO) and reactive oxygen species (ROS) are produced (Kastenbauer et al., 2002). Brain cells can also produce cytokines and other pro-inflammatory molecules in response to bacterial stimuli (van Furth et al., 1996). It maintains the cascade of host immune response through the recruitment and activation of the PMN, matrix metalloproteinase (MMPs) and other pro-inflammatory mediators that contribute to the BBB breakdown. Although, the host response is necessary to eliminate the bacteria; undoubtedly it is the major cause of brain injury (Heath and Okike, 2010).

TNF-α, IL-1β and IL-6 are cytokines with early response after pneumococcal recognition and they are associated in pneumococcal meningitis (Mook-Kanamori et al., 2011). Intrathecal administration of TNF-α resulted in a same pathophysiological feature of bacterial meningitis and BBB breakdown facilitating bacterial traversal into the CSF (Rosenberg and Li, 1995) and it also promoted IL-1β production (Nathan and Scheld, 2000). IL-6 has mainly pro-inflammatory effects and it is a mighty inducer of fever, leukocytosis and acute-phase protein (Gruol and Nelson, 1997). Furthermore, cytokine induced neutrophil chemoattractant-1 (CINC-1) is involved in the infiltration of inflammatory cells into the brain parenchyma (Katayama et al., 2009). It was produced earlier in jugular plasma than in arterial plasma in meningitis animal model (Barichello et al., 2012a, 2012b).

It maintains the cascade of host immune response through the recruitment and activation of the PMN, matrix metalloproteinase (MMPs) and others pro-inflammatory mediators that contribute to the BBB breakdown. Although, the host response is necessary to eliminate the bacteria; undoubtedly it is the major cause of brain injury (Heath and Okike, 2010).

For this purpose, we measured TNF-α, IL-1β, IL-6, IL-10 and CINC-1 levels, oxidative damage, enzymatic defense activity and the blood–brain barrier integrity in neonatal Wistar rats submitted to pneumococcal meningitis.

2. Results

In hippocampus, Fig. 1, the levels of CINC-1 and IL-1β were increased at 6 h ($p<0.01$; $p<0.05$, respectively), 12 h ($p<0.05$), 24 h ($p<0.001$) and 96 h ($p<0.001$) after pneumococcal meningitis induction. The cytokines IL-6 and IL-10 were not altered when compared with the control group and TNF-α increased at 6 h ($p<0.01$), 12 h ($p<0.001$) and 96 h ($p<0.05$).

In cortex, Fig. 2, the CINC-1 levels were increased at 6 h ($p<0.05$) and 48 h ($p<0.001$). IL-1β was increased at 6 h ($p<0.001$), 12 h and 24 h ($p<0.05$), moreover, IL-6 was increased at 0 h ($p<0.001$), 6 h ($p<0.01$) and 12 h ($p<0.01$). IL-10 and TNF-α were increased only at 6 h ($p<0.001$) and 12 h ($p<0.01$) after pneumococcal meningitis induction.

We showed in Fig. 3 the oxidative damage and enzymatic defense activity. TBARS levels were increased at 12 h and 24 h in the cortex ($p<0.05$); however, in hippocampus the levels did not change. Protein carbonyl was increased at 24 h and 48 h in the hippocampus ($p<0.05$). There was an SOD activity increase in hippocampus at 12 and 48 h ($p<0.05$) and a decrease of the SOD levels at 24 h ($p<0.05$). CAT activity was increased at 48 h in hippocampus and in cortex after pneumococcal meningitis induction ($p<0.05$). The levels in the MPO activity did not change in hippocampus and cortex after induction, Fig. 4.

The BBB integrity of hippocampus (Fig. 5A) and cortex (Fig. 5B), were investigated using Evan's blue dye extravasation. We observed the BBB breakdown, in hippocampus, within 18 h and 24 h ($p<0.05$) and in the cortex it started at 12 h until 24 h after pneumococcal meningitis induction ($p<0.05$).

3. Discussion

In this study we showed the influence of the *S. pneumoniae* in kinetic cytokine/chemokine, myeloperoxidase activity, oxidative stress and BBB integrity in two brain regions, hippocampus and cortex of neonatal rats induced by pneumococcal meningitis. The neonates brain produced higher levels of cytokine/chemokine, oxidative damage and enzymatic defense in the early times of this infection. Following this, we observed the BBB breakdown in the hippocampus and cortex of infected neonates. In other studies, TNF-α, IL-6 and IL-10 concentrations, as well as, the granulocyte infiltration showed an increase at 18 h post-infection in CSF (Sury et al., 2011), and apoptosis occurred in post-mitotic immature neurons in the dentate gyrus in experimental neonatal pneumococcal meningitis (Grandgirard et al., 2007a, 2007b). Furthermore, neonatal rats submitted by pneumococcal meningitis presented behavioral deficits in adulthood (Barichello et al., 2010b).

Bacterial meningitis is a devastating disease during the neonatal period (Huang et al., 2000), causing a complex and serious inflammation, that is associated with a high mortality rate (Hoffman and Maldonado, 2008). Nevertheless, *S. pneumoniae* can replicate rapidly and release cell components into the CSF. Bacterial cells constituent are recognized by Toll-like receptors activating the host immune response (Klein et al., 2006). In consequence, it leads to the activation of nuclear factor kappa B (NF-Kb) and triggers the expression of inflammatory cytokines and polymorphonuclear leukocytes, which

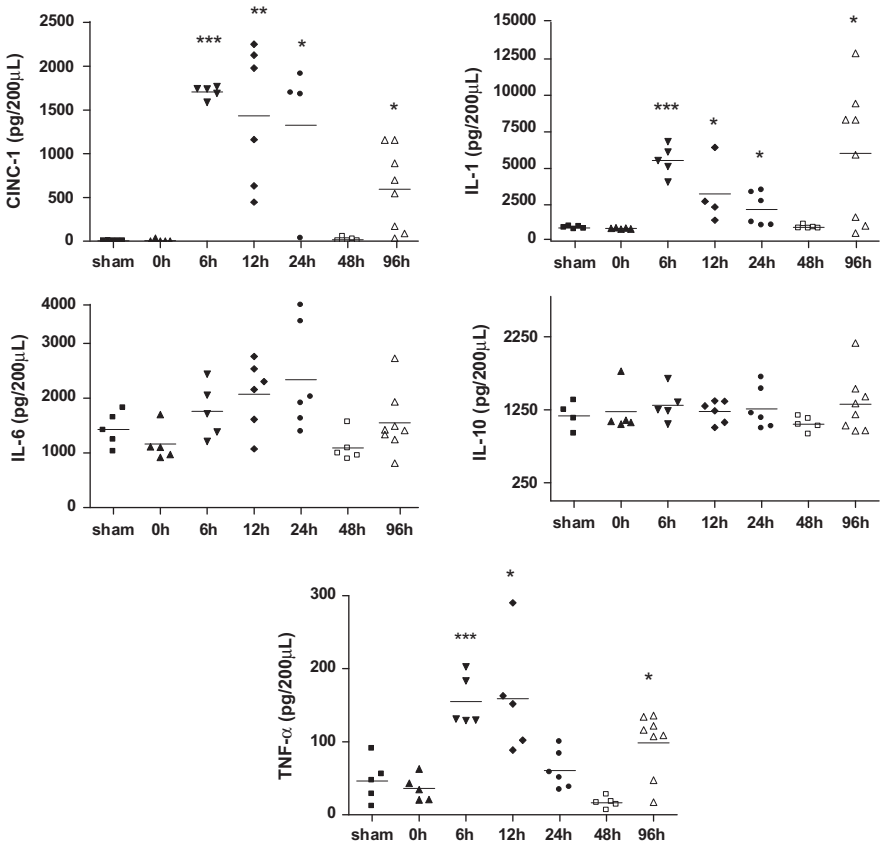

Fig. 1 – *Kinetics of CINC-1, IL-1β, IL-6, IL-10 and TNF-α expression in hippocampus after induction of S. pneumoniae meningitis*. The concentrations of CINC-1, IL-1β, IL-6, IL-10 and TNF-α were obtained in several times at 0, 6, 12, 24, 48 and 96 h after meningitis induction. Levels of cytokines/chemokine were assessed by ELISA and results are shown as pg of cytokine/chemokine per 100 mg of tissue. Results show the mean ± S.E.M. of 4–6 animals in each group. Symbols indicate statistically significant when compared with sham group $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

are attracted to the bloodstream and cross the BBB (Granert et al., 1994). As a result, large amounts of superoxide anion and nitric oxide are produced, leading to the formation of peroxynitrite (Klein et al., 2006) initiating the lipid peroxidation, oxidative protein carbonyls and oxidative stress (Zhang et al., 2002; Sellner et al., 2010). We found protein carbonylation only in hippocampus at 24 and 48 h and in cortex we verified lipid peroxidation in the first hours after induction. Furthermore, neuronal membrane lipids contain a lot of highly polyunsaturated fatty acid, mainly in cortical regions (Halliwell and Gutteridge, 2007). Oxidative stress also leads to the activation of cytokine/chemokine, matrix metalloproteinase and enhancement of neutrophil activation (Klein et al., 2006). In our study we found an increase in TNF-α, IL-1β and CINC-1 in hippocampus at 6 h, and it remained elevated until 96 h after meningitis induction. TNF-α and IL-1β, likewise, have an important role as early-response cytokines (Nathan and Scheld, 2000), and are produced after pneumococcal recognition (Porwoll et al., 1999). In patients, as well as in the animal meningitis model, the TNF-α has increased early in the illness course (Brivet et al., 2005; Barichello et al., 2010c), moreover, TNF-α administration into the CSF results in the BBB breakdown and generation of a neutrophilic inflammation (Rosenberg and Li, 1995; Tureen, 1995). IL-1β is a pro-inflammatory cytokine that mediates some changes related with bacterial meningitis, such as, fever, neutrophilia (Saukkonen et al., 1990) and it is produced by mononuclear phagocytes, glial cells in the central nervous system (CNS) through stimulation of bacterial compounds or TNF-α (Nathan and Scheld, 2000). In addition, another chemokine involved in the inflammatory cells infiltration into the brain parenchyma is the CINC-1 (Katayama et al., 2009). In this study, we verified that CINC-1 levels started to increase in the first hours in hippocampus and cortex after bacterial induction. In previous studies, we verified the increase of the CINC-1 levels at 6 h after meningitis induction (Barichello et al., 2010c). Furthermore, these levels were increased first in jugular plasma and then in arterial plasma in animal model of pneumococcal meningitis suggesting that its produced in the brain (Barichello et al., 2012a, 2012b). CINC-1 is a neutrophil chemoattractant and it may be related to early events in the pneumococcal meningitis pathophysiology. The cytokines and CINC-1 increase prior to the BBB breakdown and this breakdown occurred in hippocampus at 18 h and in cortex at 12 h after pneumococcal meningitis induction. Microorganisms can induce BBB dysfunction by affecting the release and/or expression of cytokines, chemokine and cell-adhesion molecules, which results in increased BBB permeability and pleocytosis (Kim, 2003).

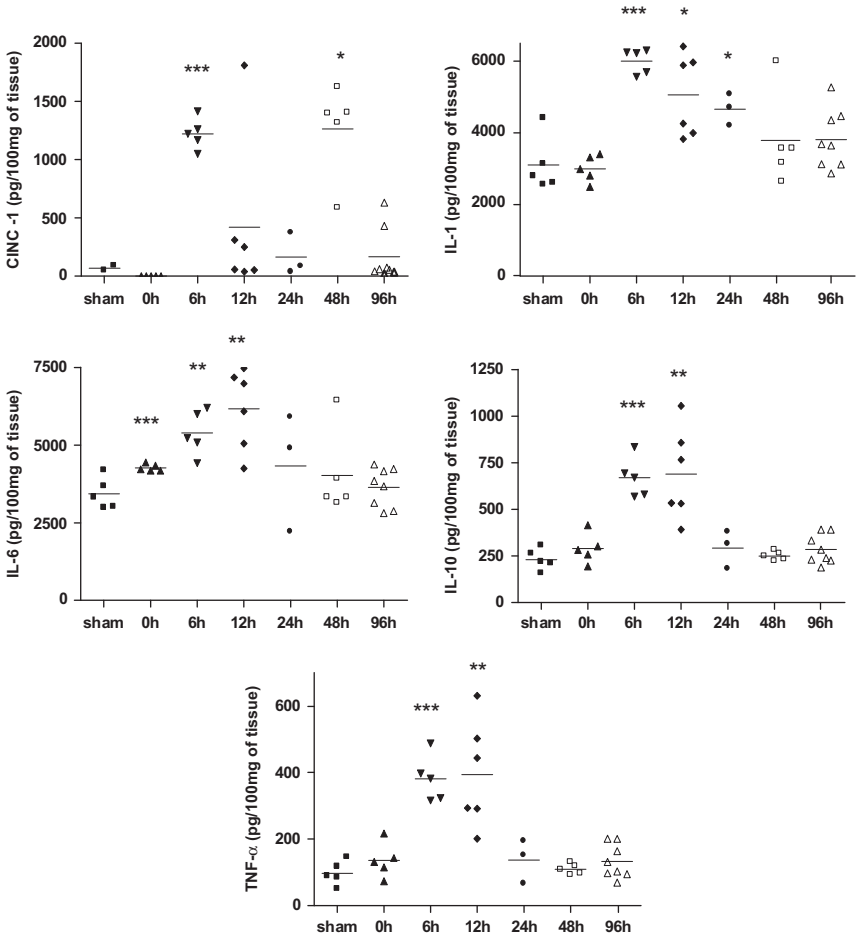

Fig. 2 – *Kinetics of CINC-1, IL-1β, IL-6, IL-10 and TNF-α expression in cortex after induction of S. pneumoniae meningitis*. The concentrations of CINC-1, IL-1β, IL-6, IL-10 and TNF-α were obtained in several times at 0, 6, 12, 24, 48 and 96 h after meningitis induction. Levels of cytokines/chemokine were assessed by ELISA and results are shown as pg of cytokine/chemokine per 100 mg of tissue. Results show the mean ± S.E.M. of 4–6 animals in each group. Symbols indicate statistically significant when compared with Sham group $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

There is still the urgent need for new adjunctive treatment strategies for bacterial meningitis (Tyler, 2008). Although descriptive, our results demonstrate that during this period, there was an increase of lipid peroxidation, protein carbonylation, cytokines and chemokines.

Neonatal bacterial infections are severe; the interference with the complex network of cytokines, chemokines, oxidants and other inflammatory mediators may be responsible for the BBB breakdown and tend to aggravate the illness.

4. Experimental procedures

4.1. *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 logarithmic phase. The culture was centrifuged for 10 min at (5000 g) and resuspended in sterile saline to the concentration of 1×10^6 cfu/mL. The size of the inoculum was confirmed by quantitative cultures (Grandgirard et al., 2007a; Barichello et al., 2010b).

4.2. *Animal model of meningitis*

Neonatal male Wistar rats (15–20 g body weight), postnatal days 3–4, 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) (Grandgirard et al., 2007a; Barichello et al., 2010a). Rats underwent a cisterna magna tap with a 23-gauge needle. The animals received either 10 μL 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., 2008; Barichello et al., 2010a). Following their recovery from anesthesia, animals were fed by their progenitor. Meningitis was documented by a quantitative culture of 5 μL of cerebral spinal fluid (CSF) obtained by puncture of the cisterna magna (Barichello et al., 2010a).

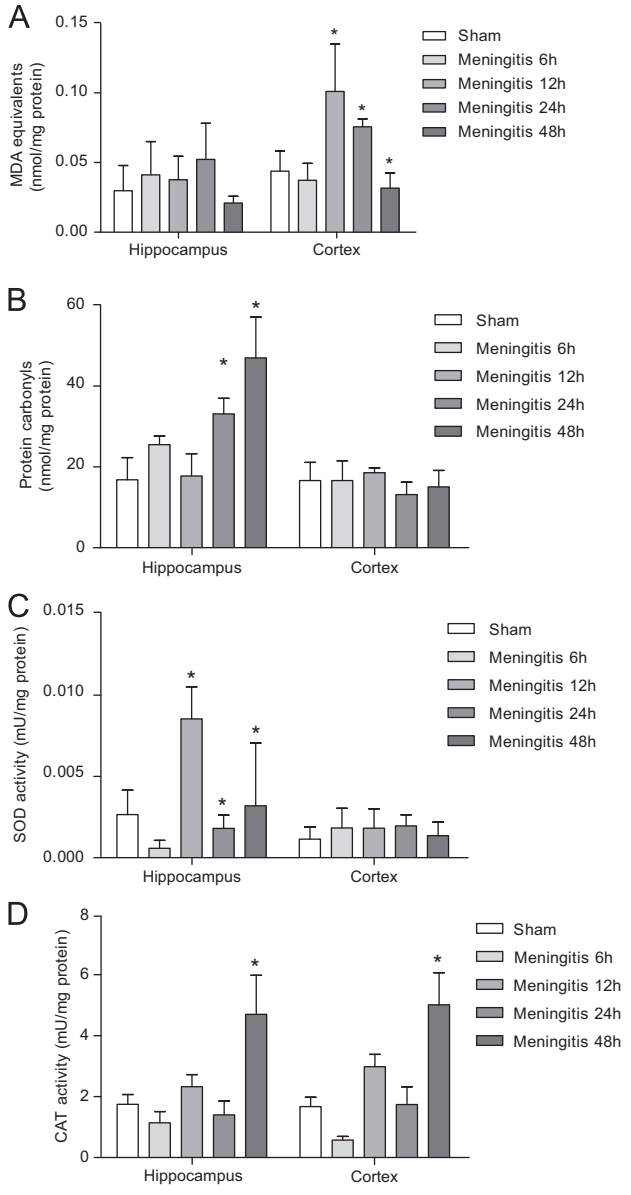

Fig. 3 – *Oxidative damage and enzymatic defense in the rat brain during development of pneumococcal meningitis*. The concentration of thiobarbituric acid reactive species (TBARS) (A); protein carbonyls (B); superoxide dismutase (SOD) activity (C); and catalase (CAT) (D) at 6, 12, 24 and 48 h after induction. Values are expressed as mean ± S.E.M. of 5 animals in each group. *Significant difference compared with Sham group $^{*}p < 0.05$.

4.3. *Assessment of TNF-α, IL-1β, IL-6, IL-10 and CINC-1 concentrations*

Animals were killed by decapitation at different times after the meningitis induction: 0, 6, 12, 24, 48 and 96 h. The brain structures hippocampus, cortex and cerebrospinal fluid were immediately isolated on dry ice and stored at −80 °C for analysis of the TNF-α, IL-1β, IL-6, IL-10 and CINC-1 levels. Briefly, hippocampus and cortex were homogenized in extraction solution containing aprotinin (100 mg of tissue per 1 mL). The concentration of cytokines/chemokine was

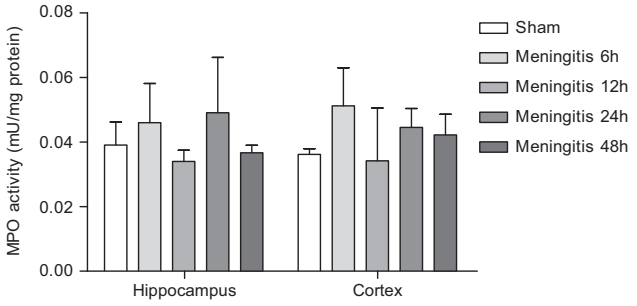

Fig. 4 – *Myeloperoxidase activity in hippocampus and cortex after S. pneumoniae meningitis induction*. The MPO activity was obtained in several times at 6, 12, 24 and 48 h after meningitis induction. Results are expressed as mean ± S.E.M. of 5–6 animals in each group. *Symbols indicate statistically significant when compared with sham group $^{*}p < 0.05$.

determined in hippocampus and 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 tissue in cortex and in hippocampus.

4.4. *Blood–brain barrier permeability to Evan's blue*

The BBB integrity was investigated using Evan's blue dye extravasation (Smith and Hall, 1996). The animals were injected with 1 mL of Evan's blue at 1% (ip) 1 h before being killed (Coimbra et al., 2007). The anesthesia consisted of an intraperitoneal administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and acepromazine (0.16 mg/kg) (Hoogman et al., 2007; Grandgirard et al., 2007a). The chest was subsequently opened and the brain was transcardially perfused with 200 mL of saline through the left ventricle at 100 mm Hg pressured until colorless perfusion fluid was obtained from the right atrium. Samples were weighed and placed in 50% of trichloroacetic solution. Following homogenization and centrifugation, the extracted dye was diluted with ethanol (1:3), and its fluorescence was determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrophotometer (Hitachi 650-40, Tokyo, Japan). Calculations were based on the external standard (62.5–500 ng/mL) in the same solvent. The EB tissue content was quantified from a linear standard line derived from known amounts of the dye and it was expressed per gram of tissue (Smith and Hall, 1996). The animals were killed at different times at 6, 12, 18, 24 and 30 h after pneumococcal meningitis induction.

4.5. *Myeloperoxidase activity*

Tissues were homogenized (50 mg/ml) in 0.5% hexadecyltrimethylammonium bromide and centrifuged at 15,000 *g* for 40 min. The suspension was then sonicated three times for 30 s. A supernatant aliquot 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 (de Young et al., 1989).

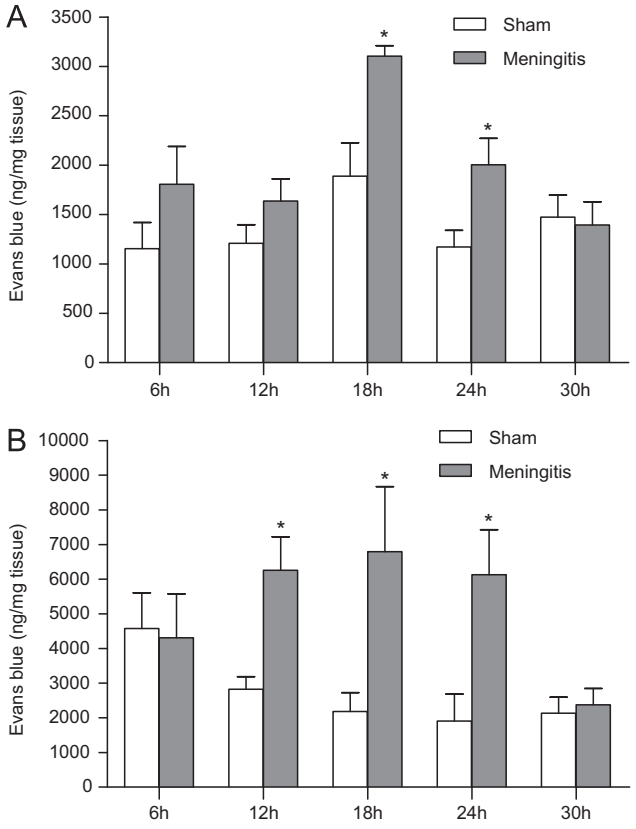

Fig. 5 – *The integrity of the blood–brain barrier was investigated using Evan's blue dye extravasation in hippocampus and cortex after induction of S. pneumoniae meningitis*. (A) Hippocampus and (B) cortex were obtained in several times at 6, 12, 18, 24 and 30 h after meningitis induction. The integrity of blood–brain barrier was assessed by fluorescence was determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrophotometer and results are shown as ng/mg tissue. Results show the mean ± S.E.M. of 5 animals in each group. Symbols indicate statistically significant when compared with sham group $^{*}p<0.05$.

4.6. *Oxidative damage and enzymatic defense activity*

Animals were killed by decapitation at different times from meningitis induction: at 6, 12, 24 and 48 h. The brain structures hippocampus and cortex were immediately isolated on dry ice and stored at −80 °C for analysis of the thiobarbituric acid reactive species (TBARS), protein carbonyls, enzymes superoxide dismutase (SOD) and calatase (CAT). The tissues 0.007 g hippocampus and 0.14 g cortex were homogenized with a phosphate buffer. The dosages were preformed immediately after homogenization and during the assays. The samples were under refrigeration and the measurements were performed in duplicate. The consistent volume of brain tissue across all the experiments, and it was determined by protein concentrations measured by the Lowry assay (Lowry et al., 1951). As an index of lipid peroxidation, we used the formation of TBARS during an acid-heating reaction (Draper and Hadley, 1990). 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 30 min. TBARS were determined based on absorbance at 532 nm. The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH), as previously described (Levine et al., 1990).

4.7. *Statistics*

The variables were shown by mean ± S.E.M. of 5–6 animals in each group. Differences among groups were evaluated by using analysis of variance (ANOVA) followed by Student–Newman–Keuls post-hoc test. For cytokine and chemokine analyses, Student's t test was used among the different hours post-infection and sham group. p values <0.05 were considered statistically significant.

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