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Experimental Neonatal Sepsis Causes Long-Term Cognitive Impairment

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Abstract Neonatal sepsis is a major cause of morbidity and mortality in neonatal intensive care units. Treatment with antibiotics reduces mortality and morbidity, but neonatal sepsis remains a serious life-threatening condition. The objective of this study was to evaluate cognitive impairment in adult mice submitted to sepsis in the neonatal period. To this aim, 2-day-old male C57BL/6 mice were submitted to sepsis by injection of 25 μg of LPS. Sixty days after, the learning and memory were evaluated. It was observed that the mice submitted to neonatal sepsis presented impairment of habituation, aversive, and object recognition memories, and had an increase of immobility time in forced swimming test in adulthood. In conclusion, this study shows that the neonatal sepsis causes long-term brain alterations. These alterations can persist to adulthood in an animal model due to a vulnerability of the developing brain.

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Introduction

Sepsis is a leading cause of morbidity and mortality in newborn and preterm infants. It is broadly defined as a systemic inflammatory response, occurring in the first 4 weeks of life as a result of a suspected or proven infection [1]. Worldwide, infections account for two thirds of the six million annual deaths in children younger than 5 years. The neonatal period has the highest lifetime risk of serious infections, with an estimated 400,000 newborn deaths annually [2].

When comparing the adult to the neonatal immune system, the latter is impaired in terms of number and functional activity of its effectors. Neutrophils and monocytes present a reduced capacity of reaching the inflammation site, and the cytokine production is decreased [3, 4]. In neonates' brain, cytokines can be released from activated immune cells. This activation is predominantly by resident microglia or activated macrophages. The immunological implications of brain immaturity, particularly with regard to the immaturity of central nervous system (CNS) immune cell regulation might render brains especially vulnerable to damage by poorly controlled and pervasive inflammation [5]. In other words, the brain of neonates is more vulnerable to damage in response to systemic inflammation.

Furthermore, late-onset sepsis in preterm infants, both microbiologically proven and clinically diagnosed, is associated with acute changes in cerebral function, shown by electrographic activity and burst suppression patterns [6]. After the neonatal intensive care unit stay, some alterations are described as persistent inflammation [7]; neurodevelopmental problems [8]; post-traumatic stress disorder [9]; depression, acute stress disorder,

and anxiety [10]; and poor quality of life [11]. Furthermore, sepsis in preterm infants, both microbiologically proven and clinically diagnosed, is associated with acute changes in cerebral function shown by electrographic activity [6]. In adults, it is known that sepsis causes long-term memory impairment in patients up to 2 years after hospital discharge [12] and in animal model up to 30 days after induction [13]. However, the pathophysiology of long-term cognitive involvement in neonatal sepsis is not yet clear.

In this context, long-term memories can be divided into associative and nonassociative. Associative memories are based on the acquisition of a predictive link between a specific event and a stimulus. Nonassociative memories are acquired when repeated or continuous exposure to a novel stimulus changes behavioral responses to it. One of the most elementary nonassociative learning tasks is that of behavioral habituation to a novel environment [14, 15]. Thus, in order to better understand the role of CNS during neonatal sepsis, the present study evaluated different types of memory (associative and nonassociative learning tasks): the habituation to the open-field (habituation memory), step-down inhibitory avoidance (aversive memory), continuous multiple trials step-down inhibitory avoidance task (learning memory), and object recognition (recognition memory). Forced swimming test (depression-like behavior) and elevated plus-maze (anxiety-like behavior) were also evaluated in adult mice submitted to sepsis in neonatal period.

Methods

Animals

Neonatal male C57BL/6 mice aged 2–3 postnatal days from our breeding colony were used for the experiments. All procedures were approved by the Animal Care and Experimentation Committee of UNISUL 13.029.4.08.IV, Brazil, and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23), revised in 1996.

Neonatal Sepsis

The animals received a subcutaneous injection of 25 μg of lipopolysaccharides (LPS) preparation in PBS (O26:B6 *E. coli* LPS; Sigma Chemical) [16]. Injection of 25 μg of LPS subcutaneously resulted in survival of 25 % after 80 h. Eighty percent of survivor animals showed a decrease of body weight and developmental delay compared to control animals that received PBS. These animals are used to conduct this study. The control group received a subcutaneous injection of PBS as a placebo in equivalent volume. At the time of the inoculation, the animals returned to their cages. Following their recovery, the animals were fed by their mothers. The animals stay with their mothers until 21 postnatal days. After that, they were separated into five animals per cage until 60 days old.

Behavioral Tasks

To evaluate the behavioral responses, the animals were separated into two groups: control and neonatal sepsis ($n=10$ per group and 20 for each behavioral task; $n=60$). Sixty days after inoculation, the animals were randomized and subjected to the following behavioral tests: (a) habituation to the open-field task, (b) step-down inhibitory avoidance task, (c) continuous multiple trials step-down inhibitory avoidance task, (d) object recognition task, (e) elevated plus-maze task, and (f) forced swimming test. Thus, using this design, we did not assess time-dependent memory, but memory over time (with new training at each test session). All behavioral procedures were conducted between 01:00 and 04:00 p.m. in a sound-isolated room, and a single animal performed only one behavior test in only one time point after surgery. All behavioral tests were recorded by the same person who was blind to the animal group.

Habituation to the Open-Field Test

This task evaluates motor performance in the training section and nonassociative memory in the retention test session. The behavior was assessed in the open field apparatus to evaluate both locomotor and exploratory activities. The apparatus is a 40×40 cm open field surrounded by 40-cm high walls made of brown plywood with a frontal glass wall. The floor of the open field is divided by black lines into 16 rectangles. Animals were gently placed on the left rear quadrant and allowed in exploring the arena for 5 min; the number of crossings (the number of times that animals crossed the black lines, assessing the locomotor activity) and rearings (the exploration behavior observed in mice when placed in a new environment) was measured. The behavioral test was performed by the same person (manual analyses) who was blind to the group treatment [17].

Step-Down Inhibitory Avoidance Task

This task evaluates aversive memory. The apparatus and procedures have been described in previous reports [18]. Briefly, the training apparatus (Insight, Brazil). In the training trial, the animals were placed on the platform and their latency to step down onto the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, the animals received a 0.2 mA, 2.0 s foot shock and were returned to their home cage. A retention test trial was performed 24 h after training (long-term memory) and was

procedurally identical to the training except that no foot shock was presented. The retention test step-down latency (maximum, 180 s) was used as a measure of inhibitory avoidance retention.

Continuous Multiple Trials Step-Down Inhibitory Avoidance Task

This task evaluates aversive memory in the test section and learning when analyzing the number of training trials required for the acquisition criterion. It was performed in the same step-down inhibitory avoidance apparatus; however, in the training session, the animal was placed on the platform and immediately after stepping down on the grid, received a 0.3 mA, 2.0 s foot shock. This procedure continued until the rat remained on the platform for 50 s. The animal was then returned to the home cage. The number of training sessions required to reach the acquisition criterion of 50 s on the platform was recorded. The retention test was performed 24 h later [18].

Object Recognition Task

This task evaluates nonaversive and nonspatial memory. The apparatus and procedures for the object recognition task have been described elsewhere [19]. Briefly, the task took place in a 40×40 cm open field surrounded by 40-cm high walls made of plywood with a frontal glass wall. The floor of the open field was divided by black lines into 16 equal rectangles. 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. The number of times the black lines were crossed and the numbers of rearings performed in this session were evaluated as indicators of locomotor and exploratory activity, respectively. At different times following habituation, training was conducted by placing individual mice in the field for 5 min. Two identical objects (objects A1 and A2, both cubes) were positioned in two adjacent corners, 10 cm from the walls. In the long-term recognition memory test that was given 24 h after training, the mice 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 of the objects had similar texture (smooth), color (blue), and size (weight 150–200 g) but with distinctive shapes. A recognition index was calculated for each animal and 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 in 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 or forepaws.

Elevated Plus Maze

The elevated plus maze consisted of two opposed open arms and two opposed closed arms, all facing a central platform which was elevated from the floor. The apparatus was placed in a small closed room illuminated by a 15-W red light (dim environment). Each mouse was placed on the central platform facing one of the closed arms. Mice were allowed to explore the apparatus for 5 min. The number of times each mouse entered into open or closed arms and the time spent in each arm was manually recorded [20].

Forced Swimming Test

The test was conducted according to previous reports [21]. Mice were individually forced to swim inside a beaker (height 18 cm; diameter 10 cm) filled with water to a depth of 12 cm. The mouse was placed in the water for 7 min (2 min for habituation and 5 min for test session). The total immobility (passive floating as opposed to struggling, climbing, and diving) duration for each mouse was manually recorded.

Statistical Analysis

Shapiro-Wilk normality test were utilized to determine the parametric and nonparametric data. Data from the habituation to the open field, the number of training trials from continuous multiple trials step-down inhibitory avoidance, the elevated plus maze, and the forced swimming tests are parametric data. It was reported as mean±SEM and analyzed by the Student's *t* test. Data from the time spent in platform for the inhibitory avoidance task and object recognition task are reported as median and interquartile ranges, and comparisons among groups were performed using Mann–Whitney *U* tests. The within-individual groups were analyzed by Wilcoxon's tests. In all comparisons, $p<0.05$ indicated statistical significance.

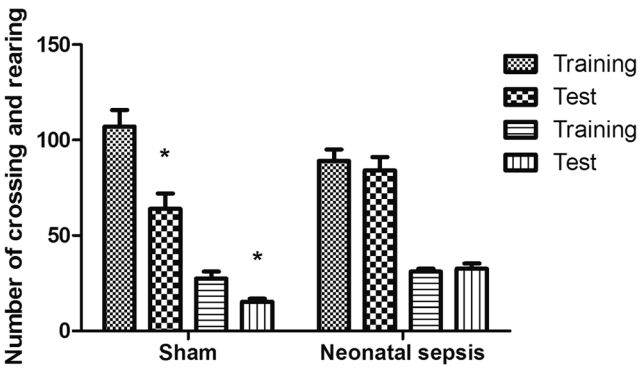

Fig. 1 Habituation on the open field. Number of crossings and rearings are presented as mean±SEM, *$p<0.05$ versus training

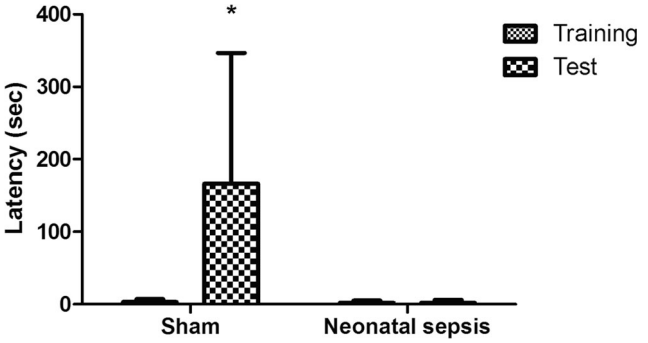

Fig. 2 The step-down inhibitory avoidance. Latency time is presented as median and interquartile ranges. *$p<0.05$ versus training

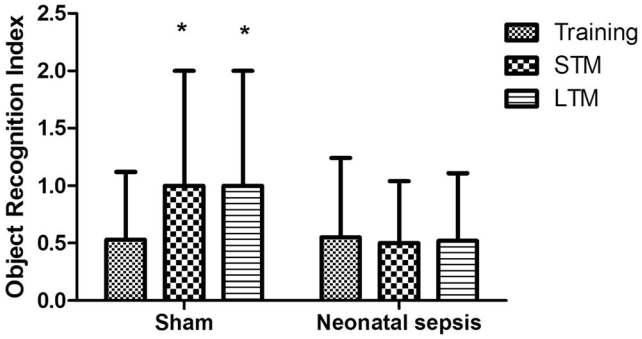

Fig. 4 Object recognition. Recognition index are presented as median and interquartile ranges, *$p<0.05$ versus training

Results

Habituation to an Open-Field Task

In the open-field task, there were no differences in the number of crossings and rearings between groups in the habituation to the open-field training session ($p>0.05$), demonstrating no difference in motor and exploratory activity between groups. In the test session, there was a significant reduction in both crossings and rearings in the test when compared with training in the sham group. In the neonatal sepsis group, there was no reduction in the number of crossings and rearings in the test when compared with training, suggesting memory impairment ($p<0.05$) (Fig. 1).

The Step-Down Inhibitory Avoidance

In the step-down latency in the test session, there was no significant difference in the latency time between training and test in the neonatal sepsis group ($p>0.05$) (Fig. 2), suggesting impaired aversive memory.

Continuous Multiple Trials Step-Down Inhibitory Avoidance Task

In the continuous multiple trials step-down inhibitory avoidance, it was demonstrated a significant increase in the number of training trials required to reach the acquisition criterion (50 s on the platform) in the neonatal sepsis group compared to the sham group ($p<0.05$) (Fig. 3a). These results suggest that the neonatal sepsis group required approximately two times more stimulus to reach the acquisition criterion when compared with the sham group, indicating learning impairment. In the retention test, there was no difference between groups for all times tested ($p>0.05$) (Fig. 3b).

Object Recognition

The neonatal sepsis group presented impairment of novel object recognition memory, i.e., they did not spend a significantly higher percentage of time exploring the novel object ($p>0.05$). The neonatal sepsis group presented memory impairment during short (STM) and the long term (LTM) (Fig. 4).

Elevated Plus Maze Task

There were no statistically significant differences in the number of entries (Fig. 5a) or in the time spent (Fig. 5b) in the arms between groups, suggesting that neonatal sepsis mice did not present anxiety-like symptoms ($p<0.05$) (Fig. 5).

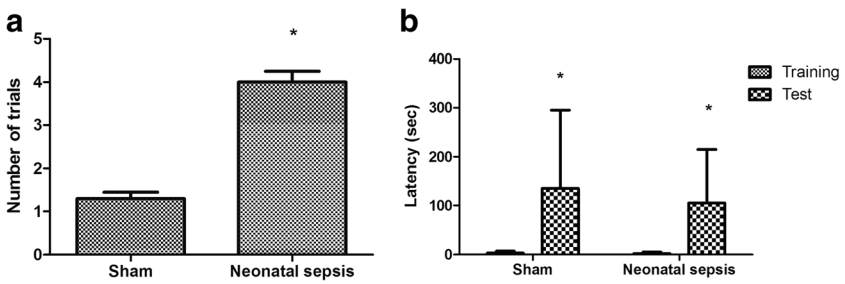

Fig. 3 Continuous multiple-trial step-down inhibitory avoidance task. **a** Training trials required to reach the acquisition criterion (50 s on the platform). Data are presented as mean±SEM, *$p<0.05$ versus sham. **b** Retention in the test session. Data are presented as median and interquartile ranges, $n=10$ mice per group. *$p<0.05$ versus training

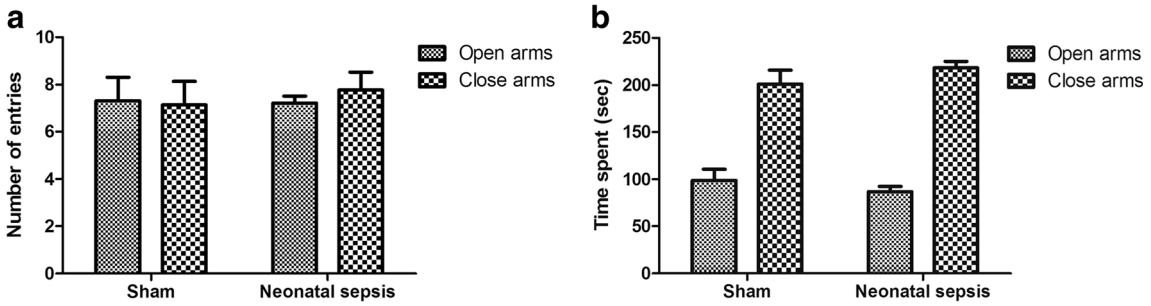

Fig. 5 Elevated plus maze test. **a** Number of entries on the closed and open arms. **b** Time spent on the closed and open arms. Data are presented as mean ±SEM

Forced Swimming Test

In the test session, we observed a significant increase in the immobility time in the sepsis group compared to the sham group ($p<0.05$), suggesting depressive-like behavior (Fig. 6).

Discussion

Neonatal sepsis is a major cause of morbidity and mortality in neonatal intensive care units [22]. While the mortality rate of neonatal sepsis has decreased, the question of the long-term sequelae of neonatal sepsis has become more important. A common feature of neonatal sepsis is the systemic activation of inflammatory mediators, which can disrupt the blood-brain barrier, interacting with the brain and thereby eliciting brain inflammation [23]. Early treatment with antibiotics reduces mortality and morbidity; however, neonatal sepsis remains a serious life-threatening condition [24, 25]. It is known that sepsis affects long-term neurodevelopmental outcomes by a sustained systemic inflammatory injury [26].

Some research show long-term complications such as neurodevelopmental and neuropsychiatric problems [8, 9]. Recent meta-analysis showed that infants who have suffered neonatal sepsis face an increased risk of mortality and severe complications such as brain damage and/or neurodevelopmental delay [27]. In this study, we showed that mice submitted to sepsis in the neonatal period present impairment in aversive, habituation, and object recognition memory, demonstrating the long-term cognitive involvement in neonatal sepsis. In addition, infants with sepsis have an increased incidence of cerebral palsy and white matter abnormalities [28, 29]. In children aged 5–16 years, it was observed that admission to intensive care is followed by deficits in neuropsychologic performance and educational difficulties, with more severe difficulties noted following septic illness [11]. In addition, the development of atopic diseases in childhood has been reported as a possible late complication of neonatal sepsis [30, 31]. In adults, cognitive impairment in sepsis has been well described both in patients and in animal models [12, 13]. However, in adult animals that survived sepsis, the cognitive behavior persisted up to 30 days after induction. In this study, it persisted up to 60 days after induction. One possible explanation for these findings is that the brain of neonates are more vulnerable to damage in response to systemic inflammation and the alterations can last for longer periods.

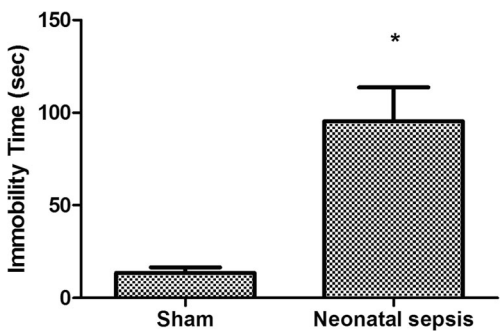

Fig. 6 The forced swimming test. They underwent the forced swimming test, and the immobility time was recorded. Data are presented as mean± SEM, *$p<0.05$ versus sham

Other interesting data obtained in this study was that neonatal sepsis causes depression-like behavior in adult mice. An increased risk of having major depression is associated with chronic disease and neurological disorders and a comorbid state of depression has been found to incrementally worsen health [32]. In this report, anxiety-like symptoms was not observed. In adult survivor patients, depressive symptoms were also relevant after ICU discharge [33], but when compared to the general ICU survivors, sepsis patients presented significantly fewer problems in the anxiety/depression dimension [34]. Adult sepsis survivor animals presented increase of immobility time up to 30 days after induction [13] and 10 days after presented depressive-like behavior [35].

The susceptibility of the CNS in neonates is associated with a fragile innate immune response. Alterations in the CNS related to inflammation and/or infection can persist to long-term period [36]. In our model, we did not study all the potential mechanisms responsible for human long-term alterations, but only the isolated effect of neonatal sepsis on neurocognitive

impairment. In addition, the concept of sickness behavior could help in elucidating some of the mechanisms associated with cognitive dysfunction after neonatal sepsis. The presented model could be a useful tool in studying cognitive impairment, depression, and anxiety neonatal sepsis, and thus the mechanisms associated with the recovery of these functions. In this context, this is the first study that shows that animal submitted to sepsis in neonatal period presented cognitive impairment and depressive-like behavior when adults. In the neonatal sepsis, inflammatory stimuli could result in brain injury via mechanisms yet unknown. These alterations can persist until adulthood in an animal model due to a vulnerability of the developing brain.

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Conflict of Interest All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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