Original Paper

Heterogeneity in the Number of Astrocytes in the Central Nervous System after Peritonitis

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ABSTRACT: Sepsis remains a major medical emergency that describes the body's systemic immune response to an infectious process and can lead to end-stage organ dysfunction and death. Clinical studies have introduced the concept of sepsis associated encephalopathy, which seems to have a plethora of cellular and molecular triggers starting from systemic inflammatory cytokines, blood-brain barrier (BBB) rupture, microscopic brain injury, altered cerebral circulation, neurotransmission, or even metabolic dysfunction. The purpose of our study is to reproduce the sepsis model previously described using the cecal ligature and puncture (CLP), and to take a closer look to the acute modifications that occur on cellular level when it comes to the brain-blood-barrier of the mice with systemic inflammation. After a rapid systemic response to peritonitis, we show a heterogeneity in astrocytic response within different cortical structures; hippocampus having the longest change in the number of GFAP+cells, while no difference was seen in the number of cortical astrocytes. With even more increasing roles of astrocytes in different pathologies, the relation between sepsis and astrocytes could prove a valuable in discovering new therapy in sepsis.

KEYWORDS: *Sepsis, atrocytic response, peritonitis.*

Introduction

Sepsis and its complications are the cause of over 50-60% of intensive care unit mortality, regardless of treatment [1]. One of the most serious complication, which further aggravate this condition, is the involvement of the central nervous system (CNS) [2], generating long lasting disabilities with in the patients that do survive [3]. As such, additional research in understanding of the neurological involvement in sepsis is needed.

The lack of specific treatment for sepsis has necessitated animal studies to identify pathological mechanisms and perhaps new markers and/or strategies of treatment. This CNS involvement seems to be a response mediated by inflammatory cytokines produced by different organs that can penetrate a disrupted but intact brain-blood-barrier (BBB) [4]. This disruption, in turn, generate a fact response from the resident immune cells of the brain, and acting as first responders [5] microglia will start to generate a local inflammatory response that will increase of the brain barrier disruption, maintaining a vicious circle of increasing CNS dysfunction and injury [6,7]. Other cells found in the CNS with a direct role in maintaining the BBB are astrocytes [8,9] and their dysfunction may exacerbate BBB permeability [10]. The activation of astrocytes in sepsis seems to be at a pick in the first 24h of sepsis [11]. In vitro tests show that astrocytic production of intracellular lactate dehydrogenase and TNF-α can induce apoptosis [12] while in vivo ones specifically link Toll-Like Receptors (TLR) to BBB leakage [7].

The purpose of our study is to reproduce the sepsis model previously described using the cecal ligature and puncture (CLP), and to take a closer look to the acute modifications that occur on the cellular level in regard to the brain-blood-barrier of the mice with systemic inflammation.

Material and Method

Mouse Model of Cecal Ligation and Puncture

Female C57BL/6 mice (54-56 weeks old, n=18), raised under specific pathogen-free conditions, were used for this study. All procedures performed were in accordance with Directive 2010/63/EU of the European Parliament and the Council and approved by the University Welfare of Experimental Animals committee (2.12/29.10.2020).

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Sepsis was induced using the cecal ligature and puncture model [13,14]. Shortly, after deep anesthesia was induced using a mix of Ketamine (120 mg/kg) and Xylazine (12 mg/kg) administered i.p) and the animals had no pinch reflex present (pinching the mice's toes using tweezers no flexion of the limb was observed). The animal was positioned on a supinated position on the operating table. The lower quadrants of the abdomen were shaved using an electric trimmer and then disinfected with a betadine solution. A longitudinal skin incision was then made with a pair of sharp scissors and the tissue between the skin and abdominal muscles was dissected to gain access to the peritoneal cavity easier. The abdominal muscles were dissected via linea alba and the peritoneal cavity was exposed. The cecum was then identified and isolated carefully in order not to damage the mesenteric blood vessels and cause a severe bleeding. The cecum position may vary according to the anatomical particularities of the animal, but it is usually located on the left side of the abdomen. The next step was to identify the ileocecal valve and to ligate the cecum at approximatively 1cm from the end. It is important to ensure that the ileocecal valve is not ligatured to maintain the intestinal continuity. A 20G needle was then used for puncturing the cecum from one side to another, resulting in two punctures of the cecum. After removing the needle, a small amount of feces was extruded from both punctures. The perforated cecum was then carefully reinserted into the abdominal cavity, in order not to touch the wound margins and contaminate them with feces.

The abdominal muscles were sutured using simple running sutures while the skin incision required simple interrupted sutures (to prevent massive opening of the wound by the animal itself) and the wound was disinfected again with betadine. With the mouse in anatomical position, 1ml of preheated saline solution was administered subcutaneous, in the lower back area, to mimic the hyper dynamic phase of sepsis.

Sham operated mice did not undergo the cecal ligature and puncture, but they were anesthetized and their abdominal cavity was exposure and then suture.

Postoperative care

After surgery, the animal was then placed inside the cage, next to a heating source and was monitored for the next 12 hours to verify easy signs of sepsis such as piloerection, fever, lethargy, diarrhea. Temperature was monitored using a rectal thermometer. A modified Murine Sepsis Scale (mMSS) [15] (Table 1) that evaluated the aspect, level of consciousness, activity, response to stimulus, eyes, and respiratory quality was determined for each animal after surgery.

Table 1. Modified Murine Sepsis Scale used in the study.

Score	0	1	2	3
Appearance	Smooth coat	Slightly ruffled fur	Back fur ruffled	Piloerection
Consciousness	Active	Avoids standing	Active only when provoked	Non-responsive, even when provoked
Activity	Normal	Suppressed eating, drinking, or running	Stationary	Stationary, even when provoked
Response to stimulus	Normal	Slowed response to auditory or touch stimuli	No response to auditory, slowed response to touch	No response to touch stimuli
Eyes	Open	Not fully open, potentially secretions	Half closed, potential secretions	Mostly or completely closed
Respiration quality	Normal	Periods of labored breathing	Consistently labored breathing	Labored breathing with gasps

Tissue preparation

After the animals were placed under deep anesthesia using a mix of Ketamine (120 mg/kg) and Xylazine (12 mg/kg) administered i.p. After intracardiac perfusion with saline solution (to remove the blood from the circulatory system) fallowed by formaldehyde for fixation, the brain and spinal cord were removed and kept in formaldehyde for 48h. After this interval, tissue was placed in paraffin blocks for histological analysis.

Immunohistochemistry

For immunohistochemistry (IHC), seriate sections were cut, deparaffinized, re-hydrated, and first processed for antigen retrieval in citrate buffer (0.1 M, pH 6) by microwaving for 20 minutes, at 650 W. After cooling down to room temperature, endogenous peroxidase was inhibited with a 1% solution of water peroxide for 30 minutes, then the unspecific binding sites were blocked in 3% skimmed milk (Biorad, California, USA) for another 30 minutes. For

enzymatic detection, the slides were next incubated for 18 hours, at 4°C, with the primary antibodies (polyclonal anti-GFAP rabbit at a 1:20.000, Dako, Denmark). Next day, the signal was amplified with a species-specific peroxidase labeled polymer (Nichirei Biosciences, Tokyo, Japan) for 1 hour, and visualized with 3,3'-diaminobenzidine (DAB) (Nichirei Biosciences). After a Hematoxylin counterstaining, the slides were cover slipped with xylene based mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Images were collected randomly from the regions of interest (cortex, hippocampus and corpus calosum), capturing three microscopic fields (671.38x562.95µm) for each region.

Statistical Analysis

After data acquisition, the number of $GFAP^+$ cells in three cortical areas (2452x2056 pixels) were counted for each mouse. The average of the counts was then further processed using Graph-PadPrism 9 and Microsoft Excel. For statistical analysis, ANOVA (Sidak test) was used to ensure more power in multiple compilations. All Figures display mean value and standard deviation (SD) and the statistical significance is displayed as follows *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

Results

Rapid systemic response to peritonitis

Although our main interest was to evaluate the astrocytic response to sepsis, some sepsis related data such as temperature and mMMS were also evaluated. Immediately after the surgery, the core temperature of the animals dropped to around 33.25±1.44oC (Figure 1A). Within the first 24 h after surgery, it steadily increased, reaching at 24 h 37.06±0.6oC. After this initial interval, the core temperature slowly increased reaching, at 72h after the surgery at 38.86±0.52oC (Figure 1A). After 24h, mMMs reached the highest score 9±0.7 (Figure 1B), showing that the immune response starts to affect the overall wellbeing of the animal. A positive correlation between core temperature of the animals and mMMS could be calculated (r=0.95) with in the first 24 h, with a negative one after (r=-0.93) (Figure 1C).

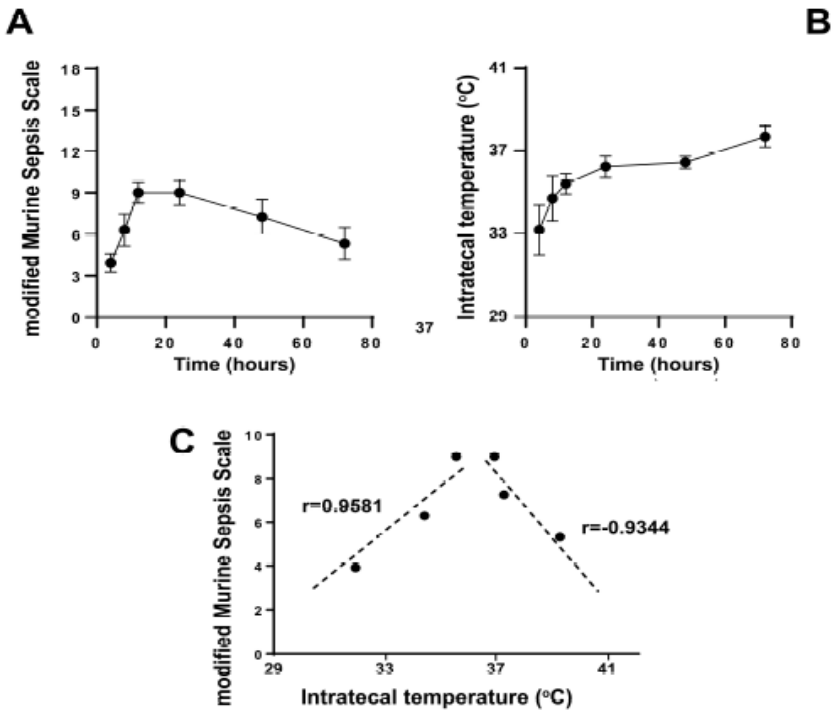

Figure 1. Systemic response to sepsis, shows that (A) within the first 24 h after CLP the overall state of the animals worsen but tends to normalize 72h after. (B) There is a constant increase in the body temperature of the animals. (C) While the initial worsening within the first 24h is positively correlated with the temperature, there is a discrepancy after that, with the improvement of the mMSS scale but with higher recorded temperatures.

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Heterogeneity in astrocytic response with in different cortical structure during sepsis

Astrocytic response proved to be extremely fast for hippocampus. Within 4h after the CLP the number of astrocytes in the hippocampus of animals that were experienced sepsis was higher compared to controls (P=0.0011, Figure 2D). This number steadily increased for the first 24h (P=0.0012) when it started to lower, and was comparable to controls 72h after the induction of CLP (P=0.16). This phenomenon was not observed for the cortex, where no difference was observed (p>0.05, Figure 2C). Interestingly, an early increase in the number of astrocytes in the corpus callosum was observed within the first 8h after sepsis (P< 0.001, Figure 2 E), but the count was fast back to normal, and remained unchanged until the end of the experiment.

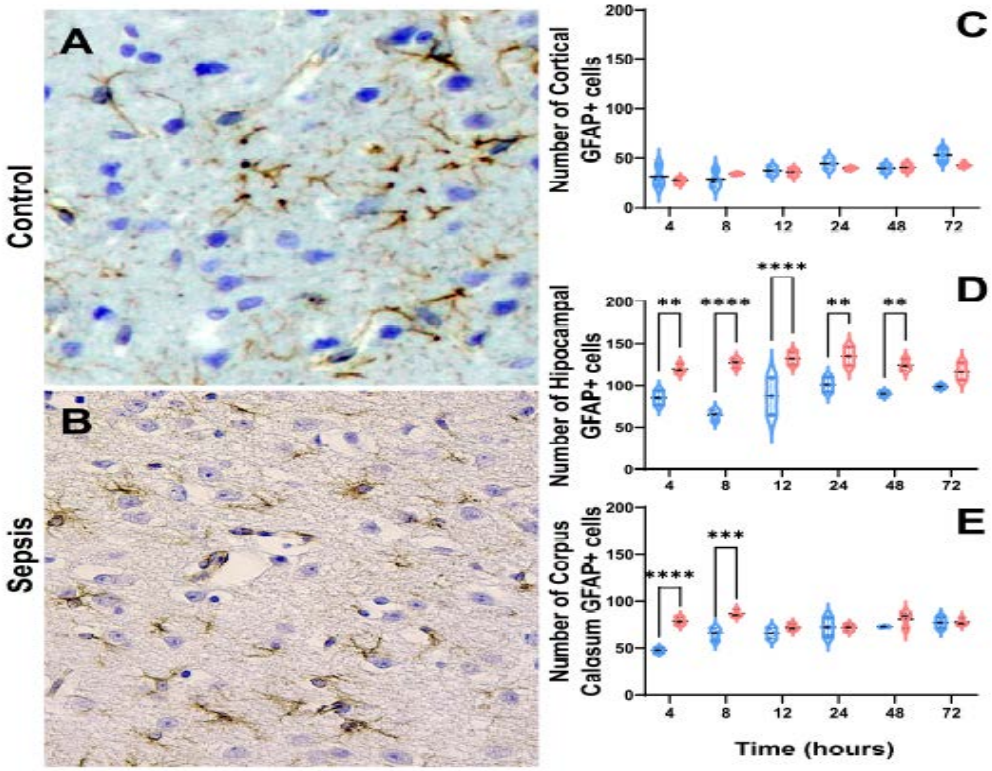

Figure 2. GFAP⁺ cells within the CNS of control (n=3) (A) and CLP (n=3) (B) animals 24h after surgery. The number of cells vary greatly in the CLP group in the hippocampus (D), while this number remains constant in the cortex of the animals (C). (E) The initial increase in GFAP⁺ cells in the corpus callosum disappear after 8h. * P ≤0.05, ** P ≤0.01, *** P ≤0.001.

Discussions

With sepsis having no specific treatment and up to 47% mortality [16] (1 si 2 pdf) one does not need additional complications. However, they occur and with the CNS ones adding to de mortality of sepsis [17,18] and having the largest impact on survivors [19,20], the need of an accurate pathological mechanism to minimize/prevent it, is increasing.

Clinical studies have introduced the concept of sepsis associated encephalopathy, which can be defined as a diffuse cerebral dysfunction, that can occur during systemic sepsis, without a direct contamination of the central nervous system [21]. The first symptom associated with this encephalopathy is disturbance of consciousness, a symptom that is easy to identify and treat, so that long-term complications are minimized [22]. Sepsis associated encephalopathy is first, characterized by altered mental status, during a systemic septic state, and it can appear anytime during sepsis, sometimes even before objectifying other systemic manifestations.

In order to investigate in more detail the connection between sepsis and it's impact on the CNS, we wanted to take a closer look at changes that occur at the cellular level in the brain of mice with systemic inflammation (CLP) in the acute phase. It is not clear if SAE is triggered by systemic inflammatory cytokines, blood-brain barrier rupture, microscopic brain injury, altered cerebral circulation, neurotransmission, or metabolism [23,24].

The BBB dysfunction theory in sepsis is not new, with one study showing that neurovascular activation and BBB degradation can be a result of inflammation originating within the CNS; for example, direct neuronal sensing of peritoneala infection could lead to CNS-intrinsic inflammation, linking TLR signaling and MyD88 in particular to the neurovascular activation and neuroinflammation during systemic infection [7].

This is specifically interesting as astrocytes can present two opposite responses to systemic toxicity: they can either rebuild their ultrastructure, activate intracellular transport and secretion mechanisms, leading to restoring impaired homeostasis or can start accumulating intracellular water, leading to the disintegration of their intracellular ultrastructure, which lead to their functional failure and cause even more tissue damage [25]. Our results show that these two processes could happen simultaneous in different parts of the CNS, as the astrocytic response is not homogenous within the brain, with deeper structures being more sensible to this systemic change than the cortex (Figure 2). Moreover, our data shows different time points of astrocytic activation, as the number of $GFAP^+$ cells were increased only in the first 8h in the corpus callosum (Figure 2E). Interesting, by looking at the cerebral blood flow and BBB in the cortex, others have seen a decrease in the number of $GFAP^+$ cells in the corpus callosum, with a altered AQP4 and neuronal COX-2 expression [26], showing that, like in other pathologies [27, 28], water balance is far more important for he CNS that previously thought. As such a strategies that showed promises in stroke [29] could also play a role in the prevention of SAE.

Furthermore, as there is a slight increase in depression among survivors of severe sepsis [30] and specific hippocampal astrocytic signaling is involved in depression [31] one must take in account the need of more detailed research on specific astrocytic sepsis involvement.

Conclusion

Sepsis remains a major medical emergency that describes the body's systemic immune response to an infectious process and can lead to end-stage organ dysfunction and death.

Despite all the medical advances made in understanding the pathophysiology of this clinical syndrome, sepsis remains a major cause of mortality among patients.

BBB is one of the most important structures of the CNS and is a target area in sepsis, requiring a thorough study of the changes that occur at this level barriers during sepsis.

There is a close link between CNS and BBB astrocytes, as they play a direct role in maintaining BBB integrity, and any dysfunction of astrocytes can exacerbate BBB permeability.

The activation of astrocytes in sepsis was at a pick in the first 24h of sepsis in the hippocampus of animals that were experienced.

However, no difference was observed for the cortex and an early increase in the number of astrocytes in the corpus callosum was observed within the first 8h after sepsis.

These data show that BBB permeability in sepsis and the connection between CNS-BBB-astrocytes remain a very good starting point for future studies, with good potential in discovering new therapies in sepsis.

Conflict of interests

None to declare.

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