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Research article

Intestinal microbiota impact sepsis associated encephalopathy via the vagus nerve

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ABSTRACT

Objective: The pathogenesis of sepsis associated encephalopathy (SAE) remains poorly understood. Vagus nerve plays an important role in gut-microbiota-brain axis. This study aimed to investigate whether vague nerve is a key mediator of the impact of intestinal microbiota on SAE.

Methods: Male rats were randomly divided into four groups (n = 20): SHAM (SH) group, lipopolysaccharide (LPS) group, fecal microbiota transplantation (FMT) +LPS group, and vagotomy (VGX) + LPS + FMT group. The left cervical vagotomy was performed 30 min before LPS administration in LPS + FMT + VGX group. LPS+ FMT and LPS + FMT + VGX groups received nasogastric infusion of feces from healthy donor three times a day. Fecal samples were collected every two days to monitor changes in microbiota composition by 16S rDNA analysis. Brain function was evaluated by behavioral tests and EEG. The levels of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-10 in brain cortex were detected by ELISA. The expression of Iba-1 in brain cortex was assessed by immunohistochemistry and Western blot analysis.

Results: Significant modification of microbiota composition, characterized by a profound increase of commensals in the Firmicutes phylum and depletion of opportunistic organisms in the Proteobacteria phylum, was observed in FMT groups compared to LPS group. Furthermore, we identified a reconstituted bacterial community enriched in Firmicutes and depleted of Proteobacteria. In both FMT groups the diversity of the fecal microbiota and the microbiota composition were similar to SH group. LPS mice treated with FMT demonstrated a better spatial memory and less EEG abnormalities, significantly attenuated levels of IL-1β, IL-6, TNF-α, and decreased number of Iba-1 positive microglia in the cortex, but these beneficial effects of FMT were reversed by VGX.

Conclusions: FMT can change intestinal microbiota in sepsis patients, and vagus nerve is a key mediator between intestinal microbiota and SAE. These findings suggest that FMT and vagus nerve are potential therapy targets for treating SAE.

1. Introduction

Sepsis remains a major clinical challenge in modern medicine and the quality of life among sepsis survivors has now been focused on their brain function [1]. In spite of the absence of direct infection of the central nervous system (CNS), sepsis survivors frequently experience significant neurological morbidity [2,3]. Brain dysfunction is a major complication of sepsis and is called sepsis-associated encephalopathy (SAE), including delirium, coma, seizure, and focal neurological signs [4–6]. Despite the high mortality rate and poor prognosis associated with SAE, effective therapy for SAE is still lacking. Currently, SAE treatments focus on controlling the systemic spread of infection and providing supportive therapy [7,8]. The pathophysiology of SAE is multifactorial and related to the effects of systemic inflammation on cerebral perfusion and neuronal activity, including inflammatory cytokines, microscopic brain injury, blood-brain barrier (BBB) compromise, and altered cerebral metabolism, neurotransmission and cerebral microcirculation [9–11]. Neuroinflammation is the main mechanisms underlying the development of SAE [12,13]. Therefore, novel therapeutic strategies for SAE aim to reduce brain inflammation.

The nervous system, via an inflammatory reflex of the vagus nerve, can inhibit cytokine release and prevent tissue injury and death [14,15]. When pathogens invade the body, inflammatory cytokines are produced and released to solitary the afferent sensory nerve, which in turn activates the efferent vagus nerve, promoting its terminus to release acetylcholine (Ach). Ach then stimulates Ach receptor on the

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surface of inflammatory cells to suppress the synthesis and release of proinflammatory cytokines, inhibiting local and systemic inflammatory responses. The cholinergic anti-inflammatory pathway is involved in the regulation of inflammation in experimental sepsis, and higher levels of vagal activity are associated with lower systemic levels of proinflammatory cytokines [16]. Vagal nerve stimulation decreased lipopolysaccharide (LPS) induced systemic TNF-α release in adult rats [17]. The role of cholinergic anti-inflammatory pathway in SAE is increasingly appreciated [18].

The dysbiosis of intestinal microbiota plays an important role in the dysfunction of the brain [19]. The anti-inflammatory vagus nerve is involved in the gut-microbiota-brain axis [20]. However, the role of vagus nerve in SAE remains largely unknown. In the present study, we aimed to examine whether the vagus nerve is a key mediator of the impact of intestinal microbiota on SAE.

2. Material and methods

2.1. Animals

Eighty adult male Sprague-Dawley (SD) rats were purchased from Hebei Medical University, Shijiazhuang, China. The protocol was approved by the Ethics Committee of Hebei Medical University, and all procedures were performed in accordance with the Guideline for the Care and Use of Laboratory Animals from the National Institutes of Health, USA. The animals were housed under a 12-h light/dark cycle in a temperature-controlled room at 24 ± 1 °C with free access to food and water.

3. Experimental procedures

SD rats were randomly divided into SH group, LPS group, LPS + FMT group, and LPS + FMT + VGX group (n = 20). Rats in all groups except SH group received intravenous injection of 10 mg/kg body weight LPS (LPS from Escherichia coli, O111:B4; Sigma-Aldrich Chemie GmbH, Germany) through femoral vein, while rats in SH group were given the same volume of saline. Severn days later, fecal samples were obtained, and the brains were removed and stored at −80 °C. For the rats in LPS + FMT + VGX groups, left vagotomy were performed 7 days before LPS administration. The left vagus nerves were exposed at the cervical level and carefully dissected from the common carotid artery. For the rats in LPS + FMT group and LPS + FMT + VGX group, fecal microbiota transplantation was performed with fresh feces from the healthy donor rats three times a day, until 7th days. The feces (3–5 g) freshly collected were diluted with sterile saline (5 ml). The homogenized solution was filtered twice through a sterilized metal sieve. The filtrates (2 ml) were infused into the rats via gavage administration on the day after LPS administration. The rat's stool was collected at 1, 3, 5, 7 days, and an aliquot (1 g) of each sample was immediately stored at −80 °C until DNA extraction.

3.1. Morris water maze test

Morris water maze test was performed in a circular pool with a diameter of 100 cm and a height of 50 cm, in an isolated environment (Jiliang Software, Shanghai, China). Different shapes were marked on the inner walls of the pool to recognize the relative position of the mouse. Water (21.5 ± 0.5 °C) containing food-grade titanium dioxide (JianghuTaibai, Shanghai, China) was filled to a height of the three quarters of the wall. The pool was divided into four quadrants and monitored with a video camera on the top. A platform with a diameter of 7 cm was place into one of the four quadrants, 1 cm below the water surface. Four group rats were trained for Morris water maze task from the 4th day to 7th day after LPS or saline administration. The rats were place on the platform for a total of 10 s and were then removed from the pool. In the subsequent training session, the rats were individually placed into each quadrant and were allowed to search the platform for a period of 60 s. If the rats were unable to reach the platform within 60 s, they would be placed on the platform for an additional 10 s. The escape latency, distance of swimming, and time spent in the target quadrant were recorded for each training process. Half an hour after the last training session on the 7th day, the rats were subjected to the probe trial with the platform removed from the pool. All rats were monitored for 60 s to observe the distance of swimming, time spent in the target quadrant, and frequency of crossing the platform.

3.2. Enzyme-linked immunosorbant (ELISA) assay

The hippocampus samples were collected for TNF-α, IL-6, and IL-1β detection at 7 days. The concentrations of TNF-α, IL-6, IL-10 and IL-1β were detected by ELISA kits according to the manufacturer's instructions. A standard curve was constructed using various dilutions of TNF-α, IL-6, IL-10 and IL-1β standard preparation. The levels of the cytokines were calculated according to standard curves.

3.3. Western blot analysis

The mice were killed by decapitation and the brains were removed for the determination of Iba-1 levels in the hippocampus at the indicated time points. Briefly, the hippocampus was homogenized on ice using immunoprecipitation buffer (10 mM Tris-HCl, pH = 7.4, 150 mM NaCl, 2 mM EDTA, and 0.5% Nonidet P-40) plus protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). The lysates were collected, centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was removed, and protein concentration was determined using the Pierce bicinchoninic acid Protein Assay kit (Pierce, Iselin, NJ, USA). Equal amounts of protein were separated on 4–12% NuPAGE Novex Bis-Tris gradient gels (Invitrogen, NY, USA) and transferred to the nitrocellulose membranes. After blocking with 5% non-fat milk for 1 h at room temperature, membranes were incubated with anti- Iba-1 antibody (1:500; Santa Cruz Biotech, Santa Cruz, CA, USA) overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Pittsburgh, PA, USA) for 2 h at room temperature. The protein bands were detected by enhanced chemiluminescence and the quantitation of bands was performed using the Image J software.

3.4. Immunohistochemistry

The brains were paraffin-embedded and cut into 4 μm sections. The sections were incubated in 0.1% H_2O_2 for 30 min to block endogenous peroxidase, followed by incubation with 1.5% normal goat serum for 30 min. The sections were then incubated overnight at 4 °C with Iba-1 antibody (Santa Cruz Biotech, Santa Cruz, CA, USA), and then washed and incubated with peroxidase conjugated secondary antibody (Dako EnVision system; Dako) for 1 h at room temperature. The sections were then washed and observed under microscope.

3.5. PCR and sequence analysis

Fecal samples were harvested and used for bacterial DNA extraction and sequencing of the V4 hypervariable region in the 16S rRNA gene.

3.6. EEG recordings and analysis

EEG was recorded at 7th days after LPS or saline administration. Standard EEG was performed using a Nihon Kohden manufactured EEG-9100J/K portable digital EEG system. EEG recordings and analysis followed the guidelines of the International Federation of Clinical Neurophysiology.