Low molecular weight heparin prevents lipopolysaccharide induced-hippocampus-dependent cognitive impairments in mice

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Abstract: Sepsis-associated encephalopathy (SAE) is a common complication after sepsis development, which is associated with the poor prognosis. However, no effective agent is currently available to treat this complication. The objective of the present study was to investigate whether low-molecular-weight heparin (LMWH) has protective effects against sepsis-induced cognitive impairments. Male mice were randomly divided into the control + vehicle, control + LMWH, lipopolysaccharide (LPS) + vehicle, or LPS + LMWH group. LMWH was administrated 30 min after the LPS administration (5 mg/kg) and daily afterward for 2 days. The survival rate was estimated by the Kaplan-Meier method. Behavioral tests were performed by open field and fear conditioning tests at day 7 after LPS administration. The levels of tumor necrosis factor alpha, interleukin (IL)-1β, IL-6, IL-10, malondialdehyde, and superoxide dismutase, Toll-like receptor 4, nuclear factor kappa B p65, inducible nitric oxide synthase, cyclooxygenase-2, occluding, high mobility group box-1, brain derived neurotrophic factor, and IBA1 positive cells were assessed at the indicated time points. LMWH attenuated LPS-induced hippocampus-dependent cognitive impairments, which was accompanied by decreased hippocampal IL-1β, malondialdehyde, Toll-like receptor 4, nuclear factor kappa B p65, inducible nitric oxide synthase, cyclooxygenase-2, high mobility group box-1, brain derived neurotrophic factor, and IBA1 positive cells, and increased occluding and brain derived neurotrophic factor levels. In conclusion, LMWH treatment protects against sepsis-induced cognitive impairments by attenuating hippocampal microglial activation, cytokine and oxidative stress production, disruption of blood-brain barrier, and the loss of synaptic plasticity related proteins.

Keywords: Sepsis, cognitive impairments, hippocampus, LMWH

Introduction

Sepsis-associated encephalopathy (SAE) is a well described complication in critical ill patients, which is associated with increased mortality and poor prognosis [1-4]. SAE represents one brain dysfunction caused by systemic inflammation evoked by the immune response to bacterial lipopolysaccharide (LPS) or other endotoxic bacterial cell wall components in the absence of direct brain infection [1-3]. The leading mechanisms thought to explain the pathogenesis of this acute brain dysfunction, such as inflammation, abnormal cerebral blood flow, and increased blood-brain barrier permeability, each involve the endothelium, a dynamic component of the blood-brain barrier (BBB) with vasomotor and biochemical properties [5]. These pathological processes are thought to arise from cytokine-mediated effects, either directly or indirectly by acting on the microvasculature, coagulation system, or the organs themselves [4, 5]. Therefore, drug that targets at some of these pathological processes may represent one option to treat sepsis-induced cognitive impairments.

Low-molecular-weight heparin (LMWH) is a naturally occurring glycosaminoglycan with both anticoagulant and anti-inflammatory activities [6]. Its anticoagulant mechanism of action depends on its ability to enhance the anti-thrombin inhibition of the critical serine proteases that is required for coagulation [7]. Low dose of LMWH was found to have protective effects by inhibiting neutrophil emigration and edema formation in a murine acute lung injury model [8]. Furthermore, the prophylactic use of either low dose (300-500 IU/h) of unfractionated heparin or LMWH is recommended in the...
Surviving Sepsis Campaign guidelines [9]. In particular, accumulating evidence has suggested that, apart from their anticoagulant capacity, LMWH can regulate a wide array of inflammatory diseases such as sepsis [10-12]. These findings have led to the suggestion that LMWH may decrease inflammatory responses and preserve the integrity of endothelium, which consequently improves LPS-induced cognitive impairments.

In the present study, we tested whether LMWH administration has protective effects against LPS-induced cognitive impairments. Since the hippocampus plays a key role in cognitive function and is vulnerable to inflammation, we tested whether LMWH administration would help modulate inflammation, preserve levels of neurotrophic regulatory factors, and attenuate cognitive impairments in an animal model of SAE.

Materials and methods

Animals

Two hundred and sixty C57BL/6 male mice (3-4 months, 25-32 g) were purchased from the Animal Center of Jinling Hospital, Nanjing, China. The study protocol was approved by the Ethics Committee of Jinling Hospital, Nanjing 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.

Experimental protocol

LPS derived from Escherichia Coli endotoxin (0111:B5, Sigma, CA, USA, 5 mg/kg) was dissolved in normal saline and injected intraperitoneally as described previously [13]. Control animals were injected with equivalent volumes (0.1 ml) of normal saline. The animals were randomly divided into the following four groups: Control + vehicle (normal saline) group; Control + LMWH group; LPS + vehicle group; or LPS + LMWH group. The flow chart of the experimental protocol is presented in Figure 1A.

Drug administration

For the dose-response study, LMWH (Sigma, St. Louis, MO, USA) at 100 IU/kg, 200 IU/kg, or 400 IU/kg was given subcutaneously 30 min after LPS administration and daily afterward for 2 days to determine the optimal dose for the prevention of LPS-induced cognitive impairments. Our data suggested that 200 IU/kg was the optimal dose effective in increasing the freezing behavior (Figure 1B). Therefore, 200 IU/kg of LMWH was used in the control + LMWH group and the LPS + LMWH group. For the control group, the same dose of normal saline was injected subcutaneously.

Behavioral and cognitive tests

All behavioral tests were performed at 14:00 p.m.-17:00 p.m. in a sound-isolated room by the instrument of XR-XZ301 (Xinruan Corporation, Shanghai Soft maze Information Technology Co., Ltd., China). All behavioral data were recorded by the same investigator who was blinded to the animal groups as described in our previous studies [14, 15].

Open field test

The open field apparatus consisted of a square, opaque acrylic container (60 cm × 60 cm × 40 cm). A video camera fixed 60 cm above the arena tracked the animals' movements. Mice were gently placed in the center of a white plastic chambers for 5 min while exploratory behavior was automatically recorded by a video tracking system (XR-XZ301, Shanghai Soft maze Information Technology Co., Ltd). Travel speed lower than 0.25 mm/s was defined as immobility. The total distance and total time traveled in the open field were recorded. After each test, the arena was cleaned with 75% alcohol to avoid the presence of olfactory cues.

Fear conditioning test

Mice were placed into the conditioning chamber (32 cm × 25 cm × 25 cm), with a stainless steel shock grid floor. The rats were allowed to explore for 3 min for habituation, then a 30 s, 80 dB, 1 kHz tone (CS), which co-terminated with a 2 s, 1.0 mA foot shock (US) was delivered through stainless steel bars by a constant current generator (Med Associates, Inc., St. Albans, VT, USA). Rats remained in the chamber for 30 s following the shock prior to return to home cage. The contextual fear memory was measured 24 h after training for 5 min by placing it back in the original training chamber to monitor freezing behavior. The cued fear mem-
Forefrontal cortex was tested 48 h after training and the rats were placed in a novel chamber with a continuous 3 min training tone presentation to monitor freezing behavior. Freezing behavior was defined as cessation of all movement except for respiration-related and slow pendulum movements. An observer blind to the treatment conditions scored the rats’ responses as freezing or no freezing every 10 s. The percentage freezing was calculated using the formula (number of freezing responses/total number of responses) multiplied by 100.

**Western blotting analysis**

The mice were killed by decapitation and the brains were removed for the determination of Toll-like receptor 4 (TLR4), nuclear factor kappa B p65 (NF-κB p65), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (Cox2), high mobility group box-1 protein (HMGB1), occludin, brain derived neurotrophic factor (BDNF), and β-actin 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 at 4°C for 10 min. The supernatant was removed, and protein concentration was determined using the Pierce bicinchoninic acid Protein Assay kit (Pierce Technology Co., Iselin, NJ) with a bovine serum albumin standard. Equal amounts of protein were electrophoretically 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 mouse anti-TLR4 (1:500; Santa Cruz Biotechnology), mouse anti-NF-κB (1:500; Santa Cruz Biotechnology), mouse anti-iNOS (1:500; Santa Cruz Biotechnology), mouse anti-Cox2 (1:500; Santa Cruz Biotechnology), mouse anti-HMGB1 (1:500; Shino-Test corporation, Kanagawa, Japan), rabbit anti-BDNF (1:500; Santa Cruz Biotechnology), and mouse anti-β-actin (1:5000; Sigma-Aldrich) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Pittsburgh, PA) for 2 h at room temperature. The protein bands were detected by enhanced chemiluminescence and the quantitation of bands was undertaken using the Image J software (NIH Image, USA).

Enzyme-linked immunosorbent assay (ELISA)

The mice were killed by decapitation and the brain tissues were isolated and washed with ice-cold physiological saline to remove the surface blood at 24 h, day 3, day 5, and day 7 after LPS administration. The hippocampus was then separated, weighed and placed in a homogenizer. The tissue was homogenized with 1 ml ice-cold physiological saline per 100 mg brain tissue. Hypothermal centrifugation was performed at 10,000 x g for 10 min and the supernatant was obtained. Standard curves including all cytokines (in duplicates) were generated using the reference cytokine concentrations supplied. The quantifications of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-10 were done by the instructions provided by Santa Cruz Biotechnology (Duoset kit; R&D Systems).

Measurement for malondialdehyde (MDA) and superoxide dismutase (SOD) concentrations

MDA and SOD levels were determined at 24 h, day 3, day 5, and day 7 after LPS administration. The level of MDA in the hippocampus, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances (Jiancheng Bioengineering Institute, Nanjing, China) as described in our previous study [17]. The malondialdehyde concentration is expressed as nmol/mg protein. SOD activity in the hippocampus was determined using a SOD assay kit (Jiancheng Bioengineering Institute, Nanjing, China) as described in our previous study [17]. The SOD concentration is expressed as U/mg protein.

Immunohistochemistry

The brains were histologically analyzed using paraffin-embedded sections (4 μm thick). The sections were incubated in PBS-Triton (PBS-T) containing 0.1% H₂O₂, for 30 min to block the endogenous peroxidase, followed by rinsing for 5 min in PBST and incubated with 1.5% normal goat serum for 30 min. The sections were then incubated overnight (4°C) using anti-active caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies, diluted 1:500 with PBS. Following extensive rinsing steps in PBS, sections were reincubated with peroxidase conjugated secondary antibody (Dako EnVision+ system; Dako) for 1 h at room temperature. IBA1, a marker of microglia activation, was performed on fresh frozen tissue (10 µm coronal sections) at the level of the hippocampus. Sections were labeled with goat anti-IBA1 (1:300, Dako, Glostrup, Denmark), after pretreatment with 1% H₂O₂/methanol (20 min), microwaving in citrate buffer (pH = 6) for 2–5 min and preblocking with normal rabbit serum. IBA1 sections were pretreated with 0.04% pepsin for 20 min before blocking. The BDNF expression was determined by rabbit anti-BDNF (1:500; Santa Cruz Biotechnology).

Statistical analysis

Statistical analyses were performed by the Statistical Product for Social Sciences (SPSS; version 16.0, IL, USA). Data are expressed as mean ± S.E.M. Differences among means were
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assessed by one-way or repeated-measures of analysis of variance (ANOVA) followed by a Tukey test as appropriate. The survival rate was estimated by the Kaplan-Meier method and compared by log-rank test. A P value < 0.05 was regarded as statistically significant difference.

Results

Survival rate

Since our previous study indicated that sepsis mice showed a high mortality rate in the first 7 days, the effect of LMWH on the survival rate of sepsis mice was evaluated within the first 7 days in the present study. As shown in Figure 1C, LMWH treatment did not affect the survival rate of sepsis mice (P > 0.05).

Chronic treatment of LMWH attenuated cognitive impairments induced by sepsis

The locomotor activity was evaluated by the open field test at 7 d after LPS administration to determine whether LPS or LMWH treatment influence the locomotor activity. There was no difference in the total distance (P = 0.537) and the time spent in the center of the arena (P = 0.247) among the four groups. In the contextual fear conditioning test, LPS treated mice displayed less freezing time compared with those in the control group, which was reversed by treatment of LMWH (P = 0.042). However, there was no significant difference in posttone freezing time in the auditory-cued fear conditioning test among the four groups (P = 0.453, Figure 2).
LMWH decreases inflammatory response, and MDA levels in the hippocampus induced by sepsis

We measured the lipid peroxidation, as indicated by MDA levels. A significant increase in MDA levels was observed in the hippocampus at day 3 after sepsis development, while the treatment of LMWH prevented the increase in the MDA levels ($P = 0.037$). However, no difference in anti-inflammatory IL-10 and antioxidant enzyme SOD was observed among groups ($P > 0.05$, Figure 3). We measured TNF-α, IL-1β, IL-6, and IL-10 expressions in the hippocampus. As shown in Figures 3-5, sepsis up-regulated the inflammatory responses, as evidenced by significantly increased IL-1β at 24 h after sepsis development when compared with
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the control and LMWH-treated mice (all \( P < 0.05 \)). On the other hand, HMGB1, a late inflammatory mediator, was significantly increased at day 7 after LPS administration. All these changes were reversed by LMWH administration.

**LMWH treatment inhibited microglia activation in the hippocampus induced by sepsis**

We measured IBA1 expressions in the hippocampus. As shown in Figure 4, sepsis activated microglia, as demonstrated by IBA1-positive cells in the hippocampus when compared with the control and LMWH-treated mice (\( P < 0.05 \)).

**LMWH treatment decreased inflammation related proteins in the hippocampus induced by sepsis**

To investigate the underling mechanism, we tested whether LMWH treatment affects inflammatory markers such as TLR4, NF-κB p65, iNOS, and Cox2 in the hippocampus. As shown in Figure 4, the TLR4, NF-κB p65, iNOS, and Cox2 levels increased significantly at 3 h after LPS administration compared with the control group (all \( P < 0.05 \)). However, LMWH treatment reversed the LPS-induced all these changes compared with the LPS + vehicle group (all \( P < 0.05 \), Figure 5).

**LMWH treatment rescued the decreased BDNF expression in the hippocampus induced by sepsis**

To investigate whether LPS induced changes of synaptic plasticity-related proteins, we examined the levels of BDNF in the hippocampus. The levels of BDNF were decreased at 7 day after LPS administration compared with the control group. LMWH treatment restored the LPS-induced down-regulation of BDNF compared with the LPS + vehicle group (\( P = 0.012 \), Figure 5). In addition, immunohistochemical study also suggested that LMWH treatment reversed the LPS-induced down-regulation of BDNF induced by LPS administration (\( P = 0.026 \), Figure 6).

**Discussion**

With the increase in patients surviving a critical illness, sepsis survivors frequently experience significant neurological morbidity, including cognitive impairments [3]. This study demon-
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strates that sepsis survivors following LPS administration developed significant hippocampus-dependent cognitive impairments, with concomitant enhanced hippocampal inflammatory responses and oxidative stress, BBB disruption, and loss of synaptic plasticity related proteins. Notably, LMWH significantly reversed all these abnormal changes.

The bi-directional communication between the immune system and the brain is necessary for the appropriate immunological, physiological, and behavioral responses to immune challenge [16]. Under pathological situations, proinflammatory mediators including IL-1β can be relayed from the periphery to the brain by the humoral pathways [4, 13]. We have selected a well-defined stimulus for the innate immunity by using LPS, which has enabled us to better identify key molecules and pathways in LPS-induced cognitive dysfunction [13]. Microglia are the resident macrophage-like cells and play an important role in the brain’s innate immunity and inflammatory responses [18]. Although microglia have essential protective roles, activated microglia can contribute to neuronal cell death through the production of cytotoxic factors, including nitric oxide, TNF-α, IL-1β, and reactive oxygen species (ROS) [18]. Accumulating evidence has demonstrated that brain dysfunction during an immune challenge are mediated, in part by actions in the brain of pro-inflammatory cytokines [19-21]. In particular, it is reported that IL-1β levels are increased in vitro and are associated with hippocampal dysfunction after sepsis development, whereas anti-IL-1 therapy appears to be able to ameliorate the associated cognitive dysfunction [20]. In addition, increased IL-6 level in the brain has been linked with severe cognitive impairments and is likely to contribute to the cognitive and neuroanatomical alternations after sepsis development [21]. On the other hand, HMGB1 is a late onset biomarker that has been shown to mediate cognitive decline in sepsis survivors [22]. TLR4, the major signaling receptor for

Figure 5. Representative micrographs of IBA1 immunolabeling in the dentate gyrus region of the hippocampus at 7 d after LPS injection. Sepsis significantly up-regulated IBA1 positive cells in the dentate gyrus region of the hippocampus, where LMWH treatment decreased IBA1 positive cells. Scale bar: 30 µm. "P < 0.05 vs. the control + vehicle group; * P < 0.05 vs. the LPS + vehicle group.
LPS, plays a vital role in LPS-induced inflammatory responses [23]. TLR4 shares the capacity to bind the intracellular myeloid differentiation factor 88 [23], which further lead to the activation of NF-κB that is responsible for the induction of various inflammatory molecules including COX-2, iNOS, TNF-α, IL-1β, and IL-6 [24]. In the present study, we demonstrated that hippocampal-dependent memory impairment was evident after 7 days after LPS administration, which was supported by previous studies that peripheral administration of LPS either as single injection or given repeatedly affects learning and memory functions [25]. Importantly, LMWH treatment was able to decrease the markers of neuroinflammation, which might account for the protective effect of LMWH against LPS-induced cognitive impairments.

In normal situations, the endothelial layer provides for an anticoagulant surface to prevent the blood from clotting, which plays a vital role in the poor prognosis of sepsis as first-line responders against invading pathogens [26]. When infectious bacteria invade the bloodstream, systemic activation of inflammation leads to cytokine release and endothelial dysfunction, resulting in consequent edema formation and organ dysfunction [27-29]. This notion is supported by the finding that disruption in the BBB integrity is prominent in the development of SAE [3]. The close interplay between inflammation and coagulation is a recognized way toward organ dysfunction and mortality in sepsis [30]. The primary mechanism responsible for this procoagulant activity, however, may be the generation of proinflammatory cytokines, especially for IL-6, IL-1β, and TNF. The BBB maintains the cerebral micro-environment by tightly regulating the passage of molecules into and out of the brain to protect the brain from microorganisms and neurotoxic substances [31, 32]. Consistently, we showed the LMWH ameliorated the breakdown of BBB, as evidenced by increased occludin expression after sepsis development. Our data were supported by previous study demonstrating that LMWH significantly reduces the brain edema, and improves the functional outcomes in focal cerebral ischemia in rats [32]. Collectively, the beneficial effects of LMWH observed in the present study might be attributed to the direct inhibition of cytokine production or a decrease in the permeability of the BBB to systemic-produced cytokines. Although it is reported that LMWH does not cross the blood brain barrier under normal conditions, we can not exclude this possibility in the context of increased BBB permeability such as sepsis. The anti-inflammatory property of LMWH is likely attributed to its anticoagulant effects, by which LMWH can improve microcirculation of organs and prevent organ dysfunction.

Synaptic transmission is essential for nervous system function, and its dysfunction is a known major contributing factor to cognitive impair-
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In support with this hypothesis, LPS has been demonstrated to impair long-term potentiation, a key cellular process of learning and memory consolidation [31]. It has been previously demonstrated that LPS reduces hippocampal levels of BDNF, a neurotrophin that plays an important role in learning and memory processes [34]. Furthermore, it has been demonstrated that inhibition of IL-1β signaling through the addition of a soluble IL-1β receptor antagonist fully prevented the synaptic deficit induced by LPS administration [35]. In line with these findings, we found that LMWH treatment rescued the reduction of BDNF levels, providing a potential mechanism by which hippocampal neuroinflammation can induce cognitive impairments induced by sepsis.

Conclusion

The present study demonstrated that LMWH attenuates LPS-induced enhanced hippocampal inflammatory responses and oxidative stress, and BBB disruption and loss of synaptic plasticity related proteins, and ultimately improved cognitive impairments. Our results support the beneficial effects of anticoagulant agents on sepsis-induced cognitive impairments and provide relevant insights into the mechanisms involved in the cognitive impairments associated with sepsis and into pharmacologic treatment to SAE.

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Disclosure of conflict of interest

None.

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