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ORIGINAL ARTICLE

Selegiline Protects Against Lipopolysaccharide (LPS)–Induced Impairment of the Blood–Brain Barrier Through Regulating the NF-κB/MLCK/p-MLC Signaling Pathway

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Abstract

Disruption of the blood–brain barrier (BBB) is an important hallmark of sepsis-associated encephalopathy (SAE). Selegiline, a selective and irreversible inhibitor of monoamine oxidase type B, has been applied for the treatment of nervous disorders. In this study, we aimed to investigate whether selegiline has a protective capacity in the impairment of the BBB in both in vivo and in vitro experiments. In a sepsis mouse model, administration of selegiline ameliorated lipopolysaccharide (LPS)–induced impairment of BBB integrity. Additionally, treatment with selegiline increased the expression of the tight junction protein junctional adhesion molecule A (JAM-A) against LPS. Also, we found that selegiline inhibited the production of the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β. In an in vitro experimental model, bEnd.3 brain endothelial cells were exposed to LPS. Results indicate that stimulation with LPS significantly increased the permeability of bEnd.3 cells and reduced the expression of JAM-A, both of which were rescued by treatment with selegiline. Additionally, selegiline prevented the activation of the NF-κB/MLCK/p-MLC signaling pathway in LPS-challenged bEnd.3 cells. These results indicate that selegiline exerted a protective effect on BBB dysfunction, which might be attributed to the inhibition of the NF-κB/MLCK/p-MLC signaling pathway. These findings provide a basis for further research into the neuroprotective mechanism of selegiline.

Keywords Sepsis · Selegiline · Blood–brain barrier (BBB) · Brain endothelial cells · Permeability · NF-κB/MLCK/p-MLC signaling pathway

Introduction

Sepsis is defined as a systemic inflammatory disease associated with life-threatening organ dysfunction resulting from a progressive infection (Salomao et al. 2019). Sepsis can induce acute and chronic changes in various systems, like the genitourinary, respiratory, central nervous (CNS), and gastrointestinal systems (Nwafor et al. 2019). Therefore, patients with sepsis usually exhibit multiple symptoms, including shortness of breath, increased heart rate, dysregulated blood pressure, fever, and pain (Nwafor et al. 2019). In the past few years, researches focusing on the treatment of sepsis have rapidly increased; however, the incidence and mortality of sepsis are still climbing (Gotts and Matthay 2016). With the goal of developing novel targeted therapeutic drugs in mind, enhancing the current understanding of the cellular pathogenesis of sepsis is still needed (Huang et al. 2019). Numerous studies have supported a regulatory role for impaired blood–brain barrier (BBB) integrity in the progression of various diseases including sepsis. BBB leakage leads to enhanced permeability of brain micro-vessels and dysfunction of brain tissue (Nwafor et al. 2019). Thus, protecting the BBB's integrity is identified as part of a short- and long-term therapeutic strategy for patients with sepsis.

Selegiline (Fig. 1A) is a selective monoamine oxidase B (MAO-B) inhibitor used in the treatment of nervous disorders including depression, Alzheimer's disease, and Parkinson's disease (Tabi et al. 2020; Birks and Flicker 2000; Fabbrini et al. 2012). Interesting findings have been published to illustrate the protective effect of selegiline on inflammation-related diseases through its anti-inflammatory

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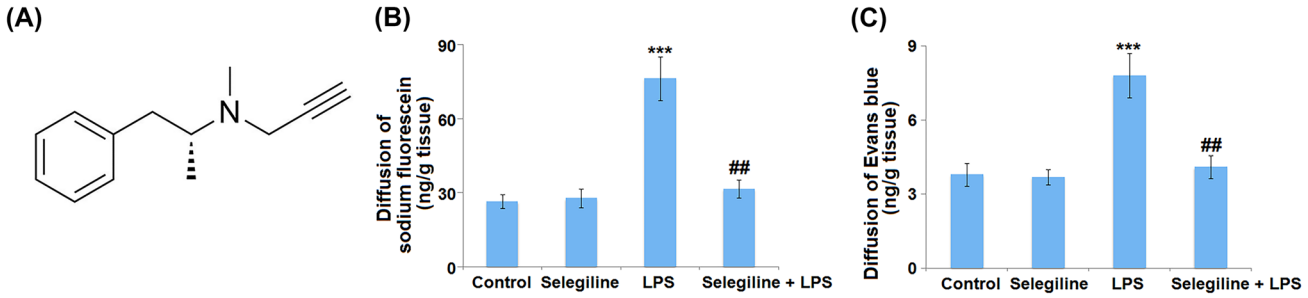

Fig. 1 Effects of Selegiline on BBB leakages in the brain of LPS-challenged mice. **A** Molecular structure of Selegiline; **B** diffusion of sodium fluorescein; **C** diffusion of Evans blue (***, $P < 0.005$ vs. vehicle group; ##, $P < 0.01$ vs. LPS group)

activity. Selegiline exhibits protective effects on cigarette smoke (CS)–mediated inflammation and oxidative stress in lung tissues from acute CS-exposed rats, driven by mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB) signaling (Cui et al. 2020). Another study also proves that selegiline reduces CS-induced oxidative and inflammatory responses in human airway epithelial cells (AECs) through Nrf2 and NF-κB signaling (Cui et al. 2017). Selegiline eliminates the aluminum phosphide (AlP)–induced inflammation and injury in the heart, duodenum, and stomach tissues of rats. Particularly, selegiline has been found to be beneficial for sepsis. In an animal model of polymicrobial sepsis, selegiline attenuates organ dysfunction and improves survival in peritonitis-induced septic rats by reducing oxidative stress, inflammation, and apoptosis (Tsao et al. 2014). However, no study has investigated the impact of selegiline on BBB integrity in sepsis. Here we intend to investigate the effects of selegiline on BBB integrity in septic rats and on brain endothelial cells permeability in vitro.

Materials and Methods

Animal Model

Protocols of animal experiments were approved by the Ethical Committee of Shanghai University of Traditional Chinese Medicine. Thirty-six C56BL/6 background mice (male, aged 8–10 weeks) were randomly assigned to four groups (9 mice per group): control group, animals received vehicles throughout the study; selegiline group, oral intake of selegiline (#ab120604, Abcam, USA) at 3 mg/kg/day for 7 days; LPS (#L2880, Sigma-Aldrich, USA) group, intraperitoneal (IP) injection of LPS at 3 mg/kg/day for 7 days; selegiline + LPS group, oral intake of selegiline at 3 mg/kg/day and IP injection of LPS at 3 mg/kg/day for 7 days. Mouse cerebral cortex tissues of three mice from each group were separated and collected for further experiments.

Quantification of the BBB Permeability

Mice were anesthetized with 1.5% pentobarbital sodium (3 mL/kg) through IP injection. Then, the mice were intraperitoneally injected with 2% sodium fluorescein (Na-Fluo, 2.5 mL/KG) (#1.03887, Sigma-Aldrich, USA) or injected with 2% Evans blue (EB; 4 mL/kg) (#E2129, Sigma-Aldrich, USA) through the tail vein using a 26 Ga catheter. Measurements of Na-Fluo and EB fluorescence were then performed as previously described (Kaya and Ahishali 2011) after 30 min and 2 h, respectively. The levels of Na-Fluo and EB were calculated using the standard curves and presented as "g/g tissue".

Immunostaining

For fixation, collected cerebral cortex tissues were fixed with 4% paraformaldehyde overnight. Then, the paraffin-embedded sections were prepared using standard protocols. Afterward, immunostaining assay for junctional adhesion molecule A (JAM-A) was conducted through incubation overnight with primary antibody against JAM-A (1:500; #ab253467, Abcam, USA) and incubation with secondary antibody (1:2000; #ab150115, Abcam, USA) for 1 h. The sections were examined using an LSM 700 META confocal microscope (Carl Zeiss).

Enzyme-Linked Immunosorbent Assay (ELISA)

The tissue homogenate of the cerebral cortex collected from experimental mice was prepared for the determination of TNF-α (#MTA00B, R&D Systems, USA) and IL-1β (#MLB00C, R&D Systems, USA) levels using commercial ELISA kits. Briefly, 50 µL of standard, control, or samples were mixed with an equal volume of assay diluent and seeded on a 96-well plate. After incubation for 2 h at room temperature, the plates were washed 3 times. One hundred microliters of Substrate Solution was then added to each well and incubated for 30 min at room temperature. One hundred microliters of Stop Solution was then added. The optical

density of each well was then measured using a microplate reader at 450 nm.

Cell Culture

The bEnd.3 brain endothelial cells (Bioleaf Biotech Co., Ltd., China) were cultured in RPMI 1640 medium (Thermo Fisher Scientific, USA) containing 15% FBS (Sigma-Aldrich, USA), 100 U/mL penicillin (Sigma-Aldrich, USA), and 100 U/mL streptomycin (Sigma-Aldrich, USA). For LPS-treated cells, bEnd.3 cells were treated with LPS at 1 μg/mL to induce inflammatory injury. For selegiline-treated cells, bEnd.3 cells were pretreated with 5 or 10 nM selegiline for 2 h, followed by treatment with LPS at 1 μg/mL for 24 h.

MTT Assay

Cell viability was assessed in bEnd.3 cells grown in a 96-well plate at the density of 1×10^5 cells per well by incubation for 4 h with MTT reagent (Sigma-Aldrich, USA) (5 mg/mL) followed by solubilization with 150 μL of dimethylsulfoxide. The absorbance at 540 nm was read and converted into a percentage of cell viability.

RT-PCR

Total RNAs isolated from cerebral cortex tissues and bEnd.3 cells were reverse transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo), and then amplified with specific primers of JAM-A, TNF-α, IL-1β using SYBR Green Master Mix (Thermo) for qRT-PCR assays. Quantification of target genes was performed by comparative Ct method with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The following primers were used in the study: JAM-A (forward,5′-CCCCGAGTGGAGTGGAAGTTCG-3′, reverse, 5′-GAGGTCTGTTTGAATTCCCCCTC-3′); TNF-α (forward,5′-GACGTGGAACTGGCAGAAGA-3′, reverse, 5′-GGCTACAGGCTTGTCACTCG-3′); IL-1β (forward, 5′- GAA ATGCCACCTTTTGACAGTG -3′, reverse 5′- TGGATGCTC TCATCA GGACAG -3′); GAPDH (forward:5′-TGACCTCAA CTACATGGTCTACA-3′, reverse, 5′- CTTCCCATTCTCGGC CTTG-3′).

Western Blot

Individual cerebral cortex tissues extract of equivalent total protein content, and whole cellular lysates and nuclear extracts of equivalent protein content were prepared and resolved by SDS-PAGE (50 μg total protein/lane). After transferring to nitrocellulose membranes, and incubating with rabbit polyclonal antibodies against primary antibody against JAM-A (1:1000; #ab253467, Abcam, USA), NF-κB p65 (1:2000, #ab207297, Abcam, USA), myosin light chain kinase (MLCK) (1:2000, #ab76092, Abcam, USA), myosin light chain (MLC) (1:3000, #ab186436, Abcam, USA), p-MLC (1:1000, #sc-12896, Santa Cruz Biotechnology, USA), β-actin (1:5000, #sc-47778, Santa Cruz Biotechnology, USA), and Lamin B1 (1:3000, #sc-377000, Santa Cruz Biotechnology, USA). Membranes were subsequently probed with HRP-conjugated anti-rabbit (1:2000, #7074, Cell Signaling Technologies, USA) or anti-mouse secondary antibody (1:2000, #7076, Cell Signaling Technologies, USA), and then the blots were visualized with enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA). Image J was used to quantify the densities of the bands.

Cell Permeability Assay

To assess the paracellular permeability, FITC-dextran fluorescein across the integrated monolayer was measured as previously described (Hu et al. 2018). The fluorescence intensity was measured using A FLUOstar Omega microplate reader with the wavelength of 485 (excitation) and 525 nm (emission), respectively.

Transendothelial Electrical Resistance (TEER) Assay

TEER assay was performed to measure the integrity of monolayer bEnd.3 cells as reported previously (Kim et al. 2020) using the Millicell ERS-2 V/ohm meter (Millipore). Based on the culture inserts, the final results of TEER level were shown as $\Omega \times cm^2$.

Statistical Analysis

The data were analyzed using the SPSS version 19.0 and presented as mean ± standard deviation (S.D.). Comparisons between multiple groups were performed by analysis of variance procedures, followed by Tukey's post hoc test. The P-value < 0.05 was considered significant.

Results

Effects of Selegiline on BBB Leakages in LPS-Challenged Mice

The assessment of BBB permeability was measured using the diffusion of Na-Fluo and EB. Compared to the control group (26.5 ± 2.8 ng/g tissue), LPS induction caused a significant increase (76.3 ± 8.9 ng/g tissue) in Na-Fluo leakage, while selegiline treatment reduced (31.6 ± 3.6 ng/g tissue) the Na-Fluo leakage in LPS-treated mice (Fig. 1B). In addition, the EB leakage in LPS-induced mice (7.8 ± 0.89 ng/g tissue) was significantly higher than that in the control mice (3.8 ± 0.46 ng/g tissue). However, the LPS-caused increased EB leakage

was reversed by selegiline treatment (4.1 ± 0.46 ng/g tissue) (Fig. 1C). The results indicate that selegiline protected the permeability of the BBB in LPS-induced mice.

Effects of Selegiline on the Expression of JAM-A in LPS-Challenged Mice

It is clear that JAM-A has a key role in maintaining the function of the endothelial barrier. To identify the regulation of JAM-A expression, we carried out qRT-PCR and immunostaining in the cerebral cortex tissues. The mRNA level of JAM-A showed a greater significant decrease (62% reduction) in LPS-induced mice, whereas JAM-A mRNA was elevated (2.0-fold increase) after selegiline administration (Fig. 2A). Consistent with this result, downregulation of JAM-A protein expression (57% reduction) in LPS-induced mice was also abolished by selegiline administration (1.67-fold increase) (Fig. 2B).

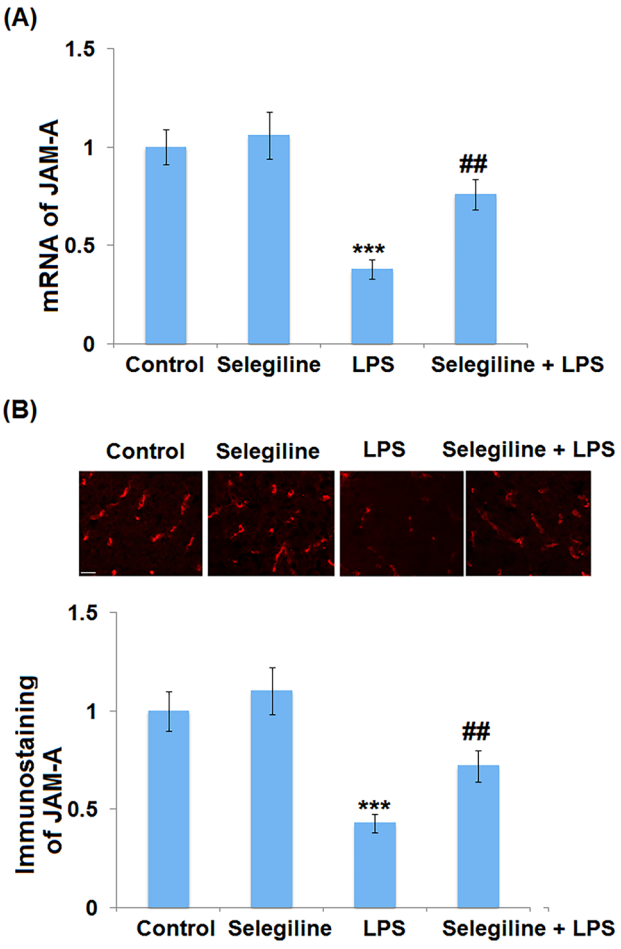

Fig. 2 Effects of Selegiline on the expression of JAM-A in the cortex of LPS-challenged mice brains. **A** mRNA level of JAM-A; **B** immunostaining of JAM-A (***, $P < 0.005$ vs. vehicle group; ##, $P < 0.01$ vs. LPS group)

Effects of Selegiline on Cytokine Levels in LPS-Challenged Mice

Next, the differences in the productions of TNF-α and IL-1β in the cerebral cortex tissues were examined by qRT-PCR and ELISA. In comparison with the control mice, LPS induced obvious increases in the mRNA levels of TNF-α and IL-1β with 4.8- and 5.9-fold changes, respectively. However, the presence of selegiline led to marked reductions in TNF-α and IL-1β mRNA levels by 45.8% and 54.2% (Fig. 3A, B). Protein levels of TNF-α and IL-1β were markedly increased in the cerebral cortex tissues from LPS-challenged mice with 3.3- and 4.8-fold changes, respectively. Moreover, selegiline administration in mice reduced the protein levels of TNF-α and IL-1β by 51.3% and 66.3% (Fig. 3C, D).

Cell Toxicity of Selegiline in bEnd.3 Brain Endothelial Cells

The cytotoxicity of selegiline on bEnd.3 brain endothelial cells was determined by recording the changes in cell viability in response to various concentrations of selegiline (0, 0.5, 1, 5, 10, 50, 100 nM). MTT assay proved that the cell viability of bEnd.3 cells was markedly reduced by 9% and 13% after incubation with 50 and 100 nM Selegiline (Fig. 4). Consistent with previous reports, the concentrations of 5 and 10 nM were used for the following experiments.

Effect of Selegiline on the Permeability of LPS-Treated bEnd.3 Cells

The protective effect of selegiline on bEnd.3 cells from LPS-induction was firstly assessed using permeability assay. The cell permeability of LPS-induced bEnd.3 cells was observed to be increased by 6.5-fold. Selegiline (5 and 10 nM) caused a 25% and 63% reduction in cell permeability of LPS-induced bEnd.3 cells, respectively (Fig. 5A). Moreover, the TEER level was found to be downregulated by 62.3% in LPS-induced bEnd.3 cells. In contrast, treatment with selegiline (5 and 10 nM) caused a significant increase in the TEER level by 1.5- and 2.3-fold, respectively (Fig. 5B).

Effect of Selegiline on the Expression of JAM-A in LPS-Treated bEnd.3 Cells

In further experiments, we tested the effect of selegiline on JAM-A expression in bEnd.3 cells. LPS decreased the mRNA level of JAM-A by 51%, which could be attenuated by selegiline (5, 10 nM) with a 1.4- and 1.8-fold change, respectively (Fig. 6A). Also, LPS produced a 47% decrease in the protein level of JAM-A, whereas selegiline (5, 10 nM) treatment increased the JAM-A protein level by 41.5% and 71.7%, respectively (Fig. 6B).

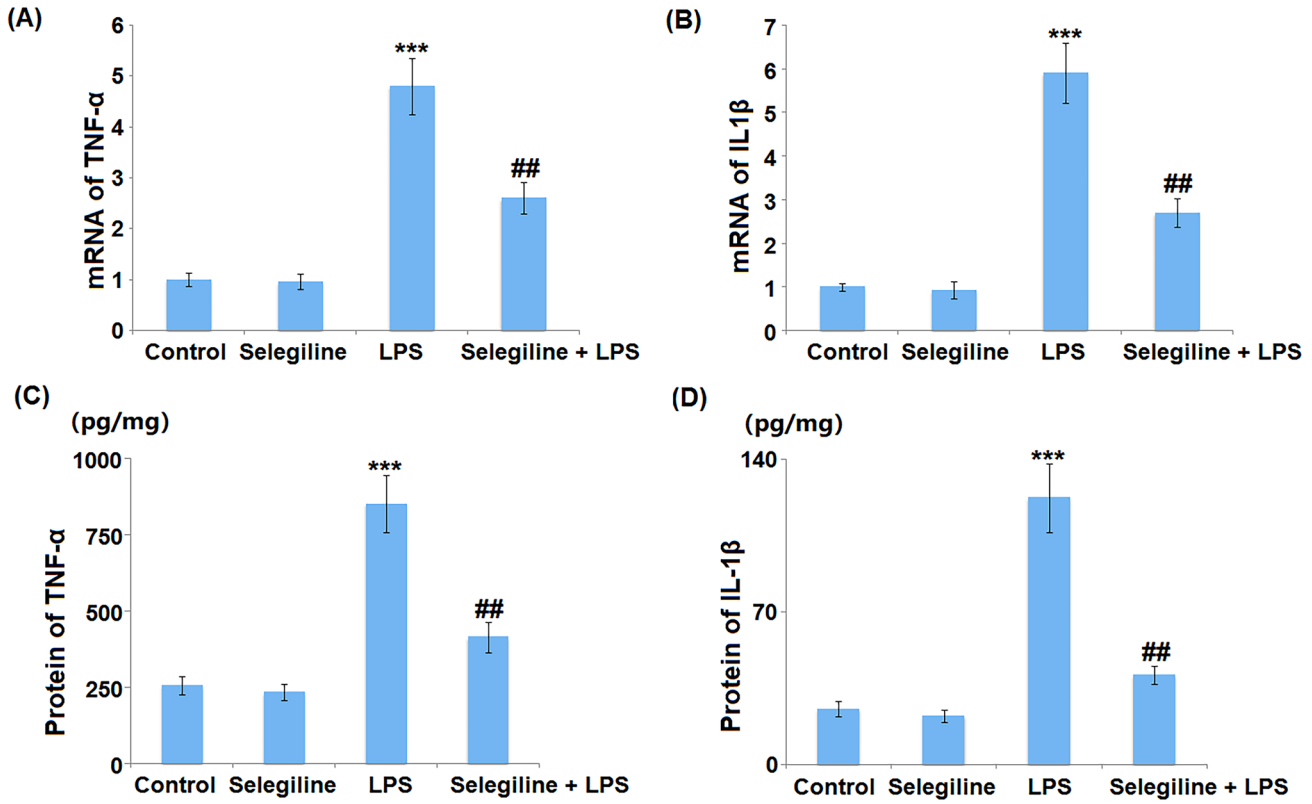

Fig. 3 Effects of Selegiline on cytokine levels in the brain of LPS-challenged mice. **A** mRNA level of TNF-α; **B** mRNA level of IL1β; **C** protein levels of TNF-α in the cortex; **D** protein levels of IL-1β in the cortex (***, $P < 0.005$ vs. vehicle group; ##, $P < 0.01$ vs. LPS group)

Effect of Selegiline on the Activation of the NF-κB/MLCK/p-MLC Signaling Pathway in LPS-Stimulated bEnd.3 Cells

Subsequently, another series of experiments were conducted to explore the signaling pathway involved in the effect of selegiline. As indicated in Fig. 7, increased protein levels of nuclear p65 (2.9-fold), MLCK (2.3-fold), and p-MLC (2.5-fold) were observed in LPS-induced bEnd.3 cells. However, there were significantly decreased protein levels of nuclear p65, MLCK, and p-MLC in the selegiline-treated bEnd.3 cells.

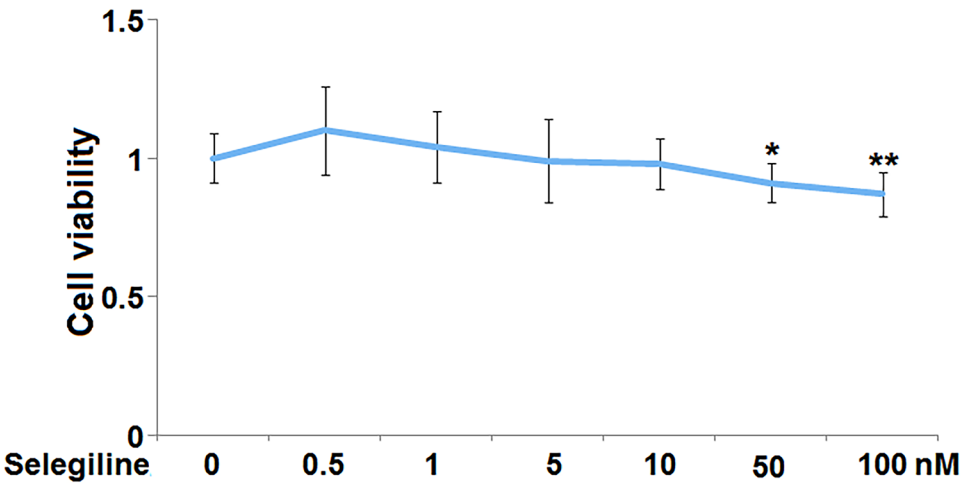

Fig. 4 Cytotoxicity of Selegiline in bEnd.3 brain endothelial cells. The cells were treated with Selegiline at various concentrations (0, 0.5, 1, 5, 10, 50, 100 nM). Cell viability was determined with MTT assay (*, **, $P < 0.05$, 0.01 vs. vehicle group)

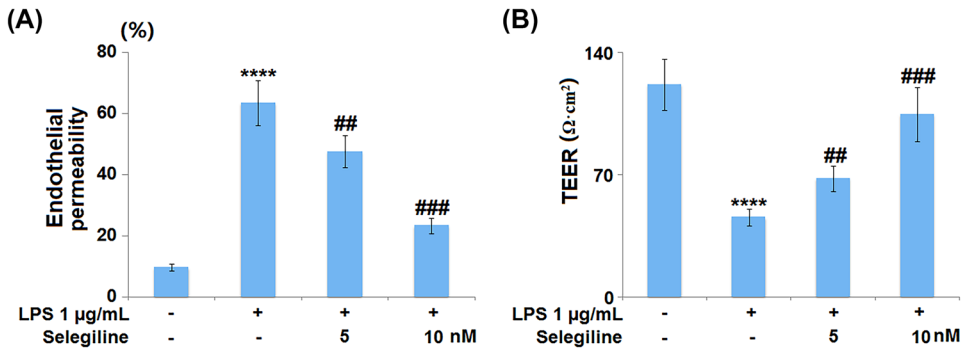

Fig. 5 Effect of Selegiline on the permeability of LPS-challenged bEnd.3 brain endothelial cells. Cells were treated with LPS at 1 μg/mL with or without Selegiline (5, 10 nM) for 24 h. **A** The cell permeability was determined by using permeability assay; **B** TEER level (****, $P<0.001$ vs. vehicle group; ##, ###, $P<0.01$, 0.005 vs. LPS group)

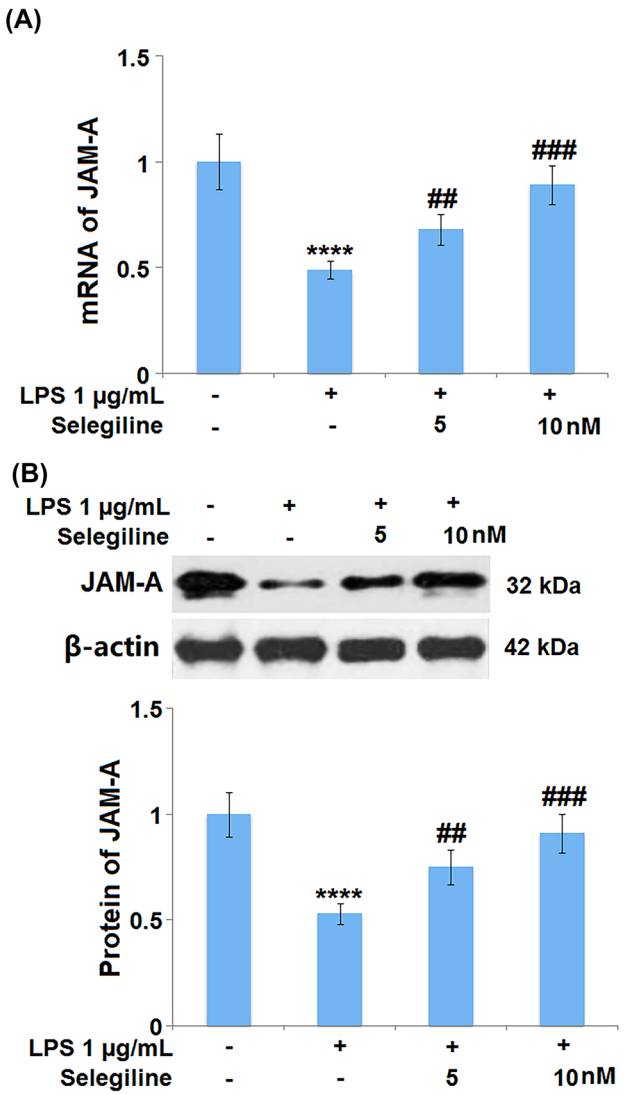

Fig. 6 Effect of Selegiline on the expression of JAM-A in LPS-challenged bEnd.3 brain endothelial cells. Cells were treated with LPS at 1 μg/mL with or without Selegiline (5, 10 nM) for 24 h. **A** mRNA level of JAM-A; **B** protein level of JAM-A (****, $P<0.001$ vs. vehicle group; ##, ###, $P<0.01$, 0.005 vs. LPS group)

Discussion

The BBB is a semipermeable biological interface between cerebral circulation and brain parenchyma, comprised of basal lamina, capillary endothelial cells, pericytes, and astrocytes (Abbott et al. 2010). Preservation of BBB integrity maintains a constant brain environment by protecting against the passage of a variety of blood-borne substances, by preventing or slowing down their passage across the BBB into the brain parenchyma. In animal models, the experimental insults used for targeting BBB integrity such as LPS have been demonstrated to increase its permeability, leading to the enhanced passage of molecules across and into the brain parenchyma. The alterations of BBB permeability can be usually assessed by the intravascular infusion of exogenous tracers, particularly horseradish peroxidase, EB dye, and Na-Fluo, followed by subsequent detection of these extravasated molecules in the brain tissues. In this study, we assessed the effect of selegiline on EB and Na-Fluo leakage in the brains of LPS-challenged mice. The results of our studies prove that selegiline protected the permeability of BBB in LPS-induced mice, as suggested by the reduced leakage of EB and Na-Fluo.

There is considerable evidence that the breakdown of endothelial tight junctions is associated with disturbances to BBB integrity (Haseloff et al. 2015; Greene and Campbell 2016), which are hallmarks of pathological alterations to the central nervous system (CNS) in sepsis (Gu et al. 2021; Gao and Hernandes 2021). Degradation of tight junction proteins contributes to the opening of the BBB, leading to fluid leaking into the brain and inducing neurotoxic effects (Berndt et al. 2019). JAM-A is a dominant tight junction transmembrane protein from the immunoglobulin superfamily (Monteiro and Parkos 2012). A potential role of JAM-A on intestinal barrier dysfunction has been indicated by a study reporting an increase in intestinal permeability and a decrease in JAM-A expression in the sepsis rat model (Haarmann et al. 2010). Cholecystokinin (CCK) protects the intestinal barrier integrity in endotoxemia rats and increases the expression of JAM-A in the colon tissues (Saia et al. 2020). Sepsis is

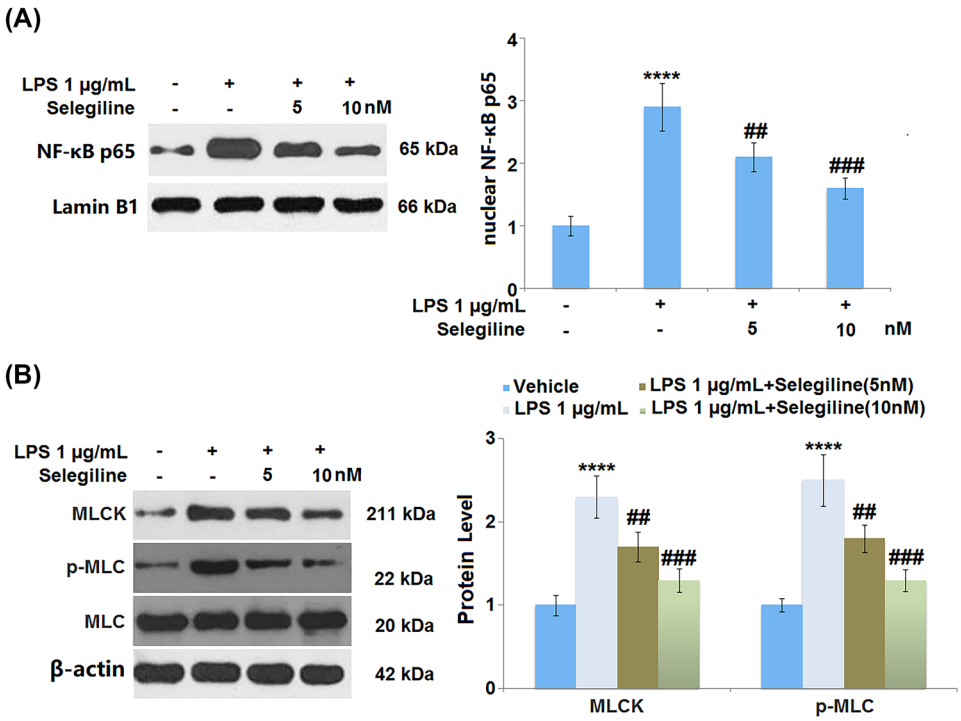

Fig. 7 Selegiline ameliorated the damage of LPS in bEnd.3 brain endothelial cells through the NF-κB/MLC/MLCK signaling pathway. **A** The protein levels of nuclear NF-κB p65; **B** the protein levels of MLCK, p-MLC, MLC as measured by western blot analysis (****, $P < 0.001$ vs. vehicle group; ##, ###, $P < 0.01$, 0.005 vs. LPS group)

associated with primary injury to the intestinal mucosa, and the previous results have demonstrated the alternations in the expression of JAM-A in the intestinal barrier. However, the involvement of JAM-A in sepsis-associated BBB dysfunction has never been reported before. Here we report that selegiline elevated the downregulated expression of JAM-A in the cerebral cortex tissues of LPS-challenged mice.

Based on the development and cell biology of the BBB, it has been indicated that the barrier-type endothelial cells in brain tissues act as significant mediators for the transcytoplasmic and paracellular trafficking of macromolecules across the BBB. Thus, endothelial cells in the brain are generally applied for studies focused on designing approaches to controlling diseases associated with BBB breakdown. In this study, exposure of the bEnd.3 brain endothelial cells to LPS was used as an in vitro model of BBB breakdown. We found that selegiline exerted a protective effect on the permeability of LPS-induced bEnd.3 cells, proven by the

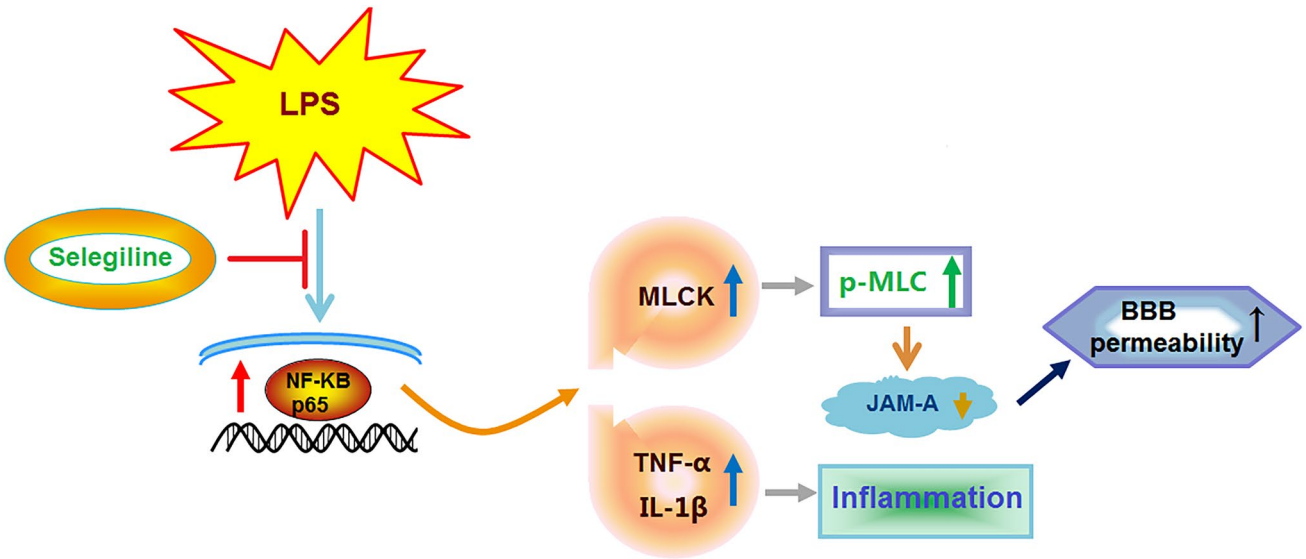

Fig. 8 Schematic illustrating the possible mechanisms of the protective role of Selegiline in BBB integrity against LPS

cell permeability and TEER assays. A previous study also suggested that the dysregulated expression of tight junction proteins is associated with the permeability of endothelial cells. Xia et al. (2020) reported that LPS exposure results in increased endothelial permeability and decreased JAM-A expression in rat pulmonary microvascular endothelial cells (PMVECs). Anticoagulant protein C presents an unexpected role in protecting against tight junction disruption (assessed by JAM-A and claudin-3 expression) and controlling epithelial barrier integrity (Vetrano et al. 2011). Our results show that alterations to the tight junction protein JAM-A's expression in LPS-induced bEnd.3 cells were prevented by selegiline, which might contribute to its protective effect on cell permeability.

It is well-known that the NF-κB transcription factor plays an essential role in inflammatory response through transcriptionally regulating the expression levels of target genes (Afonina et al. 2017). The LPS of gram-negative bacteria acts as endotoxin and binds to its receptor on the cell membrane, initiating the activation of NF-κB signaling. As a result of inflammatory mediators-dependent activation of NF-κB, expression of MLCK is increased, leading to increased phosphorylation of MLC (Shawki and McCole 2017). Increasing evidence has shown the critical role of NF-κB-mediated activation of the MLCK/p-MLC signaling pathway in an intestinal barrier and BBB dysfunction. *Escherichia coli* Nissle 1917 (EcN) protects intestinal barrier function in a septic mouse model by inhibiting the NF-κB/MLCK/p-MLC signaling pathway (Guo et al. 2019). Irbesartan suppresses LPS-induced BBB dysfunction in both in vivo and in vitro experiments by inhibiting the activation of NF-κB/MLCK/p-MLC (Yang et al. 2021). NF-κB/MLCK/p-MLC is also involved in the protective effect of anterior gradient protein 2 homologue (AGR2) on TNF-α-induced epithelial barrier dysfunction (Ye and Sun 2017). Our results aimed to explore the involvement of NF-κB/MLCK/p-MLC signaling, and we found that selegiline prevented the LPS-induced activation of the NF-κB/MLCK/p-MLC signaling pathway in bEnd.3 cells. The possible mechanisms of the protective role of Selegiline on BBB integrity against LPS are shown in Fig. 8.

In conclusion, the present study reported that selegiline exerted protective effects on BBB dysfunction in both in vivo and in vitro experiments. These effects might be attributed to the inhibition of the NF-κB/MLCK/p-MLC signaling pathway, and due to its positive role in regulating BBB function, selegiline might contribute to the treatment of BBB dysfunction-related sepsis.

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Author Contribution Yuehong Pu and Yiming Qian guaranteed the integrity of the entire study; Yuehong Pu, Fenghua Qian, Jian Guo, and Yuanyuan Sha contributed to experimental investigation and data collection; Yuehong Pu went on literature research; Yiming Qian was responsible for manuscript preparation and review. All the authors have read and agreed with the submission of the manuscript.

Data Availability The data and materials of this study are available upon reasonable request from the corresponding authors.

Declarations

Ethics Approval I confirm that all the research meets ethical guidelines and adheres to the legal requirements of the study country. Protocols of animal experiments were approved by the Ethical Committee of Shanghai University of Traditional Chinese Medicine.

Consent to Publication All the authors agreed to publish this article.

Conflict of Interest The authors declare no competing interests.

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