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Original Article

Extract of *Fructus Schisandrae chinensis* Inhibits Neuroinflammation Mediator Production from Microglia via NF-κB and MAPK Pathways*

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ABSTRACT **Objective**: To investigate the anti-neuroinflammation effect of extract of *Fructus Schisandrae chinensis* (EFSC) on lipopolysaccharide (LPS)-induced BV-2 cells and the possible involved mechanisms. **Methods**: Primary cortical neurons were isolated from embryonic (E17-18) cortices of Institute of Cancer Research (ICR) mouse fetuses. Primary microglia and astroglia were isolated from the frontal cortices of newborn ICR mouse. Different cells were cultured in specific culture medium. Cells were divided into 5 groups: control group, LPS group (treated with 1 μg/mL LPS only) and EFSC groups (treated with 1 μg/mL LPS and 100, 200 or 400 mg/mL EFSC, respectively). The effect of EFSC on cells viability was tested by methylthiazolyldiphenyl-tetrazolium bromide (MTT) colorimetric assay. EFSC-mediated inhibition of LPS-induced production of pro-inflammatory mediators, such as nitrite oxide (NO) and interleukin-6 (IL-6) were quantified and neuron-protection effect against microglia-mediated inflammation injury was tested by hoechst 33258 apoptosis assay and crystal violet staining assay. The expression of pro-inflammatory marker proteins was evaluated by Western blot analysis or immunofluorescence. **Results**: EFSC (200 and 400 mg/mL) reduced NO, IL-6, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) expression in LPS-induced BV-2 cells ($P<0.01$ or $P<0.05$). EFSC (200 and 400 mg/mL) reduced the expression of NO in LPS-induced primary microglia and astroglia ($P<0.01$). In addition, EFSC alleviated cell apoptosis and inflammation injury in neurons exposed to microglia-conditioned medium ($P<0.01$). The mechanistic studies indicated EFSC could suppress nuclear factor (NF)-κB phosphorylation and its nuclear translocation ($P<0.01$). The anti-inflammatory effect of EFSC occurred through suppressed activation of mitogen-activated protein kinase (MAPK) pathway ($P<0.01$ or $P<0.05$). **Conclusion**: EFSC acted as an anti-inflammatory agent in LPS-induced glia cells. These effects might be realized through blocking of NF-κB activity and inhibition of MAPK signaling pathways.

KEYWORDS neuroinflammation, microglia, *Fructus Schisandrae chinensis*, nuclear factor-κB, mitogen-activated protein kinase

Diverse neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis, are all characterized by various inappropriate inflammatory responses along with tissue destruction. Evidence is accumulating that neuroinflammation plays a key role in the heterogeneous pathogenesis of neurodegenerative disorders.[1]

Microglia is the main immune cell in central nervous system (CNS) and participating in neuroinflammation. Evidences have shown that microglia elicited opposite outcomes on neuron survival depending on different temporality and magnitude of activation. For example, microglia could release a trace of neurotrophic factor to protect neurons when nicotinic receptor was stimulated. Whereas stimulation by lipopolysaccharide (LPS), microglia could release a mass of pro-inflammation mediators such as nitrite oxide (NO) and interleukin (IL)-6 and lead to inflammatory cascade.[2] Substantial evidence indicated that astrocyte-mediated inflammatory responses

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also influenced development of CNS diseases. Similar to microglia, astrocytes activation may either be supportive to neurons, or instead promote disease progression, but its exact role still need further research.[3,4] The inhibition of over-activated glia is considered as a fundamental strategy to reduce neuroinflammation injury in neurodegenerative diseases.

NF-κB as the major transcription factor triggering inflammation response could regulate the expression of genes encoding various mediators.[5] Incorrect regulation of NF-κB has been linked to cancer, autoimmune diseases as well as inflammation.[6-10] The mitogen-activated protein kinase (MAPK) pathway is a family of serine/threonine-specific protein kinases including c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK). The MAPKs play a vital role in the transmission of extracellular signals into intracellular targets and are involved in directing inflammation responses to a diverse array of stimuli.[11,12] Thus, NF-κB activation and MAPKs pathways are commonly targeted with anti-inflammatory drugs.

Schisandrae Fructus, the dried fruit of *Schisandra chinensis* (Turcz.) Baill, is a traditional Chinese medicine, officially listed as a tonic in the Chinese Pharmacopoeia. At present, herbal therapy is a trend in the management of chronic diseases and the optimal utilization of herbal resource is important for a sustainable development of herbal medicine. Modern scientific research have indicated that extract of *Fructus Schisandrae chinensis* (EFSC) and its lignin compounds possessed a wide spectrum of biological activities, such as hepatoprotection, anti-inflammation, anti-oxidation and protective effect on renal ischemia-reperfusion injury.[13,14] Several studies have been conducted on the anti-inflammatory activity of *Schisandrae Fructus*, but the role of EFSC in neuroinflammation is still obscure.[15] The study was designed to investigate the anti-neuroinflammation effect of EFSC and the potential mechanism which was involved.

METHODS

Materials

EFSC was obtained from Xi'an Runlang Bio-technology Co., Ltd. (Shaanxi, China, lot No. 20141014). LPS from *Escherichia coli*, serotype 055:B5 (lot No. 046M4045V), 4',6-diamidino-2- phenylindole (DAPI, lot No. 034M4031V), crystal violet and hoechst 332589 (lot No. BCBQ5908V) were purchased from Sigma Chemical Co., Ltd. (St Louis, MO, USA). MTT (lot No. 1212J051) was from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Dulbecco's modified eagle medium (DMEM, lot No. G2512200), DMEM/nutrient mixture F-12 (DMEM/F12, lot No. F08UE030), radio-immunoprecipitation assay (RIPA, lot No. MP015) were purchased from M&C Gene Technology Ltd. (Beijing, China). Fetal bovine serum (FBS, lot No. P30-3302) and Western chemiluminescent horseradish peroxidase (HRP) substrate (lot No. PD197915) were obtained from Thermo Scientific (Waltham, MA, USA). IL-6 enzyme-linked immuno sorbent assay (ELISA) kit was purchased from ExCell Bio company (Shanghai, China). NO assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Dimethylsulfoxide (DMSO, lot No. 20150522) was purchased from Beijing Chemical Works (Beijing, China). All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

High Performance Liquid Chromatography/ Electrospray Ionization Tandem Mass Spectrometry Analysis

An Agilent series 1200 high performance liquid chromatography (HPLC) instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a diode-array detector, an autosampler and a column compartment was used for analyses. The chromatographic separation was performed on an AichromBond-AQ C18 column (250 mm × 4.6 mm i.d., particle size 5 μm, Abel Industries Ltd., Canada), which was protected by a Phenomenex C18 guard cartridge (4 mm × 2 mm i.d., particle size 5 μm, Torrance, CA, USA). The mobile phase consisted of ACN (A) and 0.1% aqueous formic acid (B), and was delivered in gradient as follows: 0–15 min, 10%–25% A; 15–20 min, 25%–50% A; 20–45 min, 50%–80% A; 45–50 min, 85–100% A; 50–55 min, 100% A; flow rate, 1.0 mL/min. At the end of each run, 100% A was allowed to flush the column for 5 min and 10% A was delivered for the subsequent 15 min to re-equilibrate the entire system. The column temperature was maintained at 35 ℃.

An Aglient 6320 ion trap mass spectrometer (Agilent, Waldbronn, Germany) was connected to the Agilent 1200 HPLC instrument via ESI interface for HPLC-mass spectrometry (MS) analysis. The LC eluent was roughly split in a ratio of 3:1 (v/v) before entering the ion source from 0 to 55 min for each sample. Ultrahigh pure helium (He) was used as the

collision gas and high purity nitrogen (N2) as the nebulizing gas. The optimized MS parameters were set as follows: positive ion mode; ion spray voltage, 4.5 kV; nebulizer pressure, 30 psi; drying gas flow, 10.0 L/min; dry temperature, 350 ℃; scan ranges, m/z 100–1100 for MS1, m/z 50–1000 for MS2, and m/z 50–900 for MS3. Data acquisition and analysis were achieved using Data Analysis software version 6.1.

Cell Culture and Grouping

The mouse BV-2 cell lines (No. 3111C0001 CCC000063) were obtained from Peking Union Medical College, Cell Bank (Beijing, China). Cells were maintained in high-glucose DMEM medium containing 10% (v/v) heat-inactivated fatal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 ℃ in a humidified atmosphere with 5% CO_2.

Primary microglia and astroglia were isolated from the frontal cortices of newborn Institute of Cancer Research (ICR) mouse [specific pathogen-free grade, from Beijing Vital River Laboratory Animal Technology Co., Ltd., license No. SCXK (Jing) 2011-0011].[16] In brief, the cortex tissues were resected from the brains and cut into 1 mm × 1 mm × 1 mm fragments, followed by digested in 0.2% trypsin for 20 min at 37 ℃. The mixed glial cells were seeded in a poly-L-lysine-coated 75 cm^2 flask and incubated with DMEM/F12 containing 10% FBS for 2 weeks. Microglias were isolated by shaking off loosely adherent cells from mixed glia cultures, while the remaining cells were mostly astroglia. The experiments were performed with the approval of the Institutional Animal Care and Use Committee of Peking University First Hospital (Approval No. 201411). Animals were housed to a room with controlled conditions of temperature (25 ℃), humidity (50%) and 12 h light-dark cycles. All efforts were made to ameliorate animal welfare and minimize suffering.

Cells were divided into 5 groups: control group, LPS group (treated with 1 μg/mL LPS only) and EFSC groups (treated with 1 μg/mL LPS and 100, 200 or 400 mg/mL EFSC). Drug concentrations were based on pilot experiments and previous researches.[15] The lowest anti-inflammatory drug concentration was 100 mg/mL in BV-2 cells.

Treatment of Neuronal Cultures with Microglia-Conditioned Medium

Primary cortical neurons were isolated from embryonic (E17-18) cortices of ICR mouse fetuses.[14] Briefly, the cortex tissues were resected from the brains and cut into 1 mm^3 fragments, followed by digested in 0.2% trypsin at 37 ℃ for 20 min. The dissociated neurons were seeded onto poly-L-lysine coated culture plates with DMEM containing 10% FBS for 4 h, then replaced the DMEM medium by neurobasal medium containing B27. After 7 days of culture, the neurons were exposed to microglia-conditioned medium. Neurons were identified with immunofluorescent staining using the antibody against neuron specific marker microtubule-associated protein 2.[17]

After BV-2 cells were treated with LPS (1 μg/mL) with or without EFSC (100, 200 and 400 mg/mL) for 6 h, the medium containing drugs were replaced by neurobasal medium containing B27. After culture with activated BV-2 cells for 24 h, the neurobasal medium was used as conditioned medium and incubated with isolated neurons to establish a microglia-neuron co-culture. After another 24 h of culture, the neurons were used in subsequent assays.

Cell Viability Assay

Cell viability was evaluated using the MTT colorimetric assay. After treatment, cell culture supernatants were replaced by medium containing MTT (0.5 mg/mL) for 2 h at 37 ℃. Subsequently, the medium was removed and the formazan crystals formed were dissolved in DMSO. Then the absorbance was detected at 570 nm. Cell viability was expressed as the percentage of the control.

NO and IL-6 Assay

The productions of NO in the supernatants were determined for nitrite by Griess method.[18] Briefly, cell culture supernatants were collected and reacted with Griess reagent (1% sulfanilamide/0.1% naphthyl ethylene diamine dihydrochloride/2% phosphoric acid) in a 1:1 ratio. Subsequently, the optical density was measured at 540 nm with a microplate reader. Sodium nitrite was used as a standard curve in the assay. After cells were treated with LPS (1 μg/mL) with or without EFSC (100, 200 and 400 mg/mL) for 8 h, the supernatants were collected for IL-6 assay. IL-6 concentrations were measured using commercial ELISA kits according to the manufacturer's protocol.

Western Blot Analysis

After treatment, BV-2 cells were washed with

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PBS and lysed in RIPA buffer (50 mmol/L Tris-HCl, 300 mmol/L NaCl, 0.5% TritonX-100, 5 mmol/L ethylene diamine tetraacetic acid, cocktail protease inhibitor) on ice for 20 min. The cell lysate was centrifuged at 13,000 r/min for 20 min at 4 ℃. The total proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked by 5% skim milk and incubated with primary antibodies overnight at 4 ℃. After incubation with a HRP-labeled secondary antibody at room temperature for 1 h, the protein immuno-complexes were developed with enhanced chemiluminescence and visualized using a Digital Imaging System (Gel Logic 2200 Pro, Kodak, Tokyo, Japan).

Immunofluorescence Assay

BV-2 cells were treated with LPS (1 μg/mL) with or without EFSC (100, 200 and 400 mg/mL) for 2 h. Then, the cells were fixed with cold 4% paraformaldehyde for 20 min at room temperature, followed by permeabilized with 0.5% TritonX-100 for 30 min and blocked with 5% bovine serum albumin (BSA) in phosphate buffered solution Twen-20 for 1 h at room temperature. Then, the cells were incubated with a primary antibody against NF-κB p65 (1:250) overnight at 4 ℃. After washing, secondary antibody conjugated to Alexa 488 (1:1000) was added and incubated for 1 h. Furthermore, the cells were stained with DAPI (5 mg/mL in PBS) for 20 min at 37 ℃ in dark. All images were taken using a fluorescence microscope (IX73, Olympus, Japan).

Hoechst 33258 Apoptosis Assay

After treatment with conditioned medium for 24 h, the neurons were fixed with 4% paraformaldehyde for 20 min at room temperature, followed by staining with Hoechst 33258 solution for 10 min at 37 ℃. The cells were then washed with PBS and images were obtained with a fluorescence microscope.

Crystal Violet Staining Assay

The neurons were washed and fixed by cold 4% paraformaldehyde at room temperature after treated with conditioned medium for 24 h. Then, 0.5% crystal violet solution was added to the cells, followed by incubation for 30 min at room temperature. Cells were washed with PBS, and images were obtained with an optical microscope (IX73, Olympus, Japan).

Statistical Analysis

Data were presented as the mean ± standard deviation ($\bar{x} \pm s$) for each group. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test with SPSS version 16.0. $P<0.05$ was considered to be statistically significant.

RESULTS

HPLC-ESI-MSn Analysis of EFSC

A total of 11 components were tentatively assigned by matching the empirical molecular formula and CID fragmentation patterns with the information documented in previous studies.[19,20]

EFSC Down-regulates Inflammatory Response in LPS-Induced Glia Cells

LPS (1 μg/mL) in the absence or presence of EFSC (100, 200 and 400 mg/mL) did not induce any cell toxicity (Figure 1A). Thus, this concentration range was used in the following experiments. As shown in Figures 1B and C, 400 mg/mL EFSC could inhibit increased production of NO and IL-6 resulting from LPS stimulation in BV-2 cells with a concentration-dependent manner ($P<0.01$). Western blot analysis showed that 400 mg/mL EFSC treatment significantly reduced iNOS and COX-2 expression in a dose-dependent manner ($P<0.01$, Figures 1D–F).

EFSC (100, 200 and 400 mg/mL) did not influence primary glia viability (Figures 2A and C). Then it was found the markedly increased production of NO induced by LPS (1 μg/mL) for 48 h in primary microglia and astroglia were reversed by EFSC treatment ($P<0.01$, Figures 2B and D).

EFSC Protects Neurons from Microglia-Mediated Inflammatory Injury

The cell viability assay indicated that LPS treatment could result in significant neuron death through microglia-mediated inflammation injury. Treatment with 100, 200 and 400 mg/mL EFSC could obvious mellow this damage ($P<0.01$, Figure 3A). Furthermore, the effect of only EFSC on neurons showed that EFSC (100, 200 and 400 mg/mL) had no effect on neuron viability (Figure 3B).

Moreover, hoechst 33258 staining results indicated obvious neuron apoptosis induced by LPS were almost reversed by treatment with 100, 200 and 400 mg/mL EFSC (Figure 3C). Crystal violet staining results showed neurons were suffered inflammation

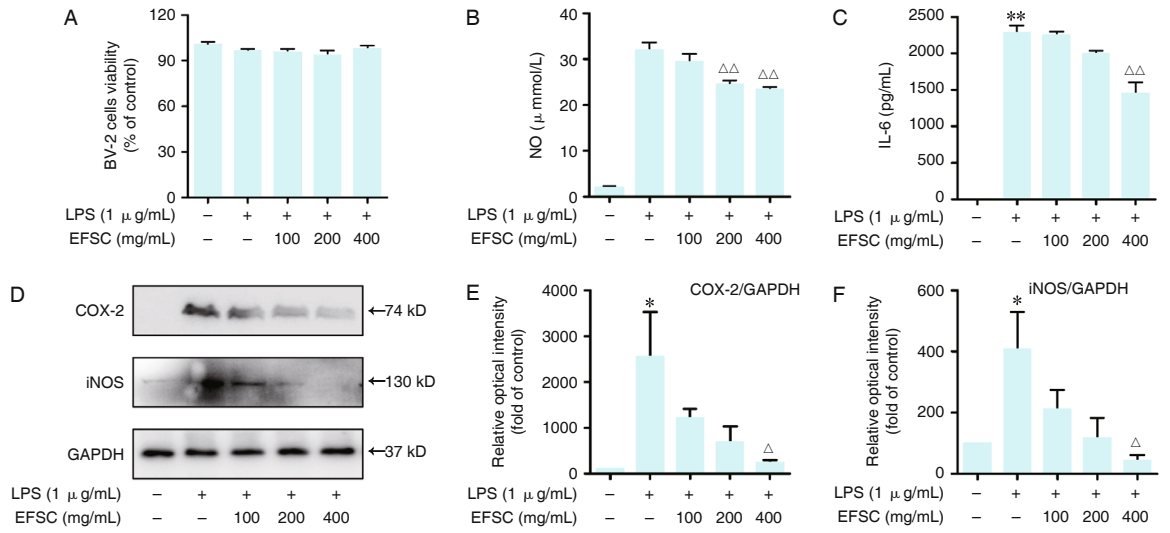

Figure 1. EFSC Inhibits Inflammation Response in LPS-Induced BV-2 Cells by Down-Regulating Expression of Inflammatory Mediators

Notes: MTT assay of cell activity (A) and detection of NO (B), IL-6 (C), iNOS and COX-2 expression (D–F) in LPS-induced BV-2 cells treated with or without EFSC (100, 200 and 400 mg/mL) for appropriate time. $^{*}P<0.05$, $^{**}P<0.01$, *vs*. control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, *vs*. LPS group

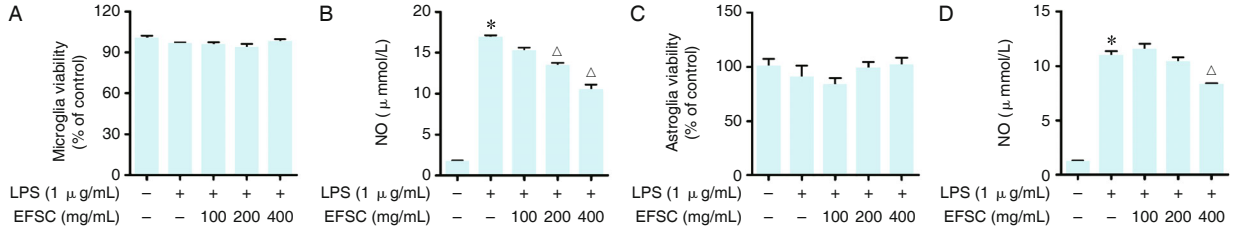

Figure 2. EFSC Inhibits Inflammation Response in LPS-Induced Primary Miroglia and Astroglia Cells by Down-Regulating NO Expression

Notes: A–B: primary microglia; C–D: primary astroglia; MTT assay of cell activity (A) and detection of NO (B) in primary microglia induced by LPS with or without EFSC (100, 200 and 400 mg/mL) for 48 h. MTT assay of cell activity(C) and detection of NO (D) in primary astroglia induced by LPS with or without EFSC (100, 200 and 400 mg/mL) for 48 h. $^{*}P<0.01$, *vs*. control group, $^{\triangle}P<0.01$, *vs*. LPS group

injury induced by LPS with significant neurite loss and cleavage, however, EFSC could significantly alleviate the inflammation injury (Figure 3D).

EFSC Inhibits NF-κB Activation in LPS-Induced BV-2 Cells

EFSC (100, 200, 400 mg/mL) significantly down-regulated phosphorylation level of NF-κB p65 induced by LPS ($P<0.01$, Figures 4A and B). Furthermore, the accumulation of NF-κB p65 in the nucleus induced by LPS was alleviated by EFSC (100, 200, 400 mg/mL) treatment according to immunofluorescence assay (Figure 4C).

EFSC Inhibits MAPKs Pathway Activation in LPS-Induced BV-2 Cells

MAPKs pathway activation were down-regulated by 200, 400 mg/mL EFSC in a dose-dependent manner ($P<0.01$ or $P<0.05$, Figure 5).

DISCUSSION

Accumulating evidence indicated that inflammatory processes were involved in neurodegeneration diseases and might be both the cause and the consequence of the disorder.[21] During the progress of neuroinflammation, glial cells actively participate in inflammatory responses by releasing proinflammatory mediators which play a vital role in the disease pathogenesis. NO, a major iNOS-derived product, is an important cellular signaling molecule involved in responses to various stimulants and could trigger inflammatory response.[22-24] IL-6 is another important component in the initiation and development of inflammatory cascade. Our results supported a significant inhibition of LPS-induced NO and IL-6 production by EFSC without notable cytotoxicity. In addition, EFSC could attenuate the increased expression of iNOS and COX-2 induced by LPS treatment in BV-2 cells. Furthermore, in the co-culture system of primary

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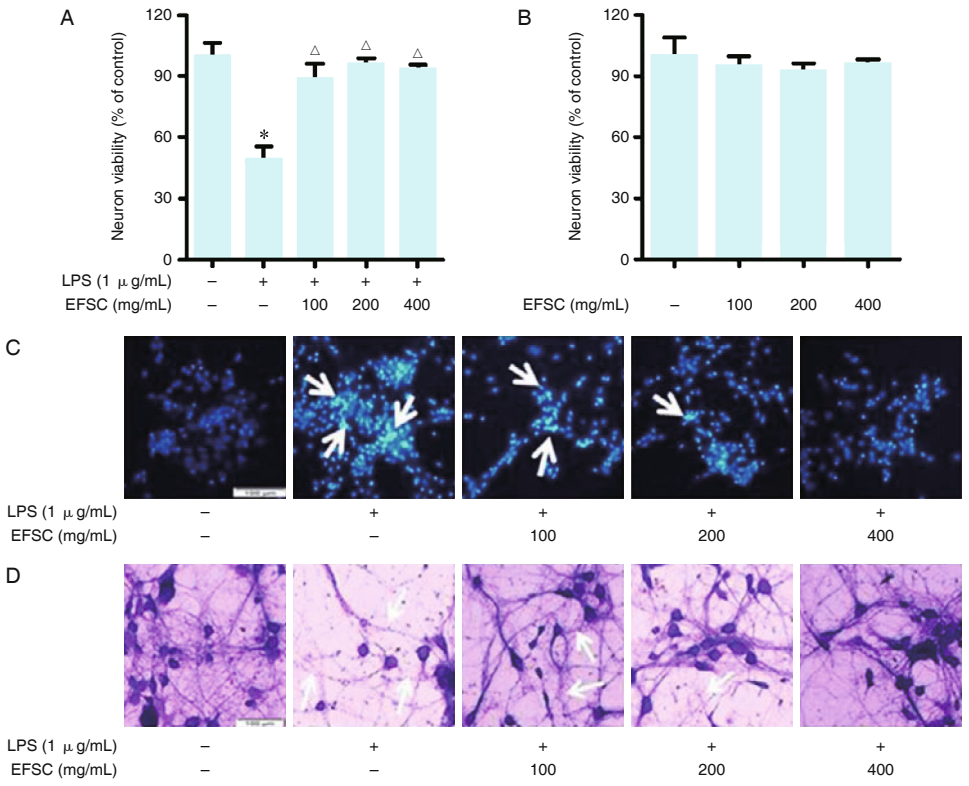

Figure 3. EFSC Protects Neurons from Microglia-Mediated Inflammatory Injury

Notes: A: MTT assay of neuron activity in microglia-neuron co-cultures induced by LPS with or without EFSC (100, 200 and 400 mg/mL) for 24 h. B: MTT assay of neuron activity treated with EFSC (100, 200 and 400 mg/mL) only. Hoechst 33258 staining (C) and crystal violet staining (D) of neurons in microglia-neuron co-cultures induced by LPS with or without EFSC (100, 200 and 400 mg/mL) for 24 h. The arrows in the C diagram indicate the apoptotic neurons. The arrows in the D diagram indicate neuronal synapses (bar = 100 μm); *P<0.01, *vs*. control group, △P<0.01, *vs*. LPS group

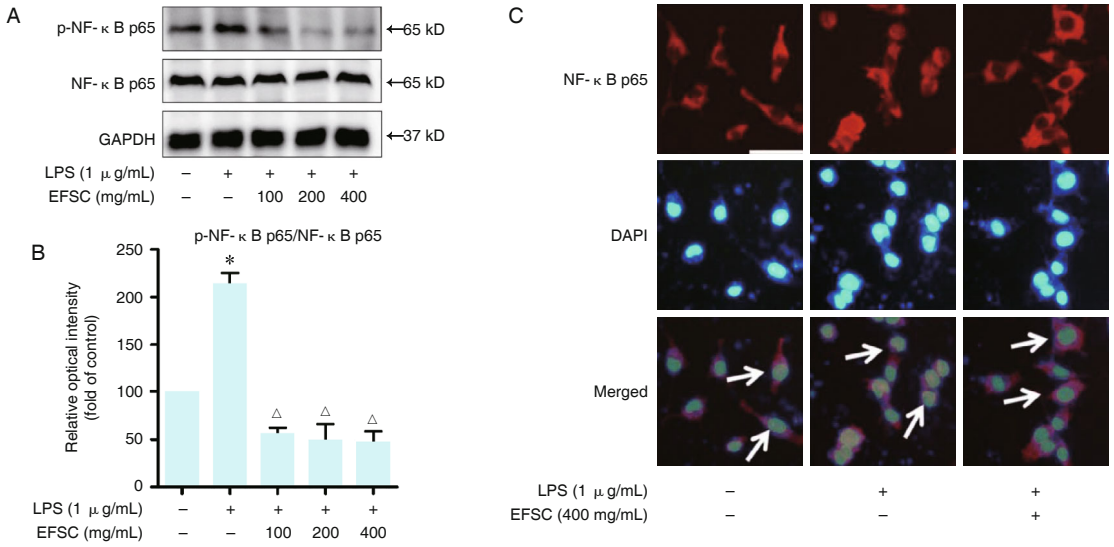

Figure 4. EFSC Inhibits NF-κB Activation and Nuclear Translocation in LPS-Induced BV-2 Cells

Notes: (A–B) Western blot analysis of p-NF-κB p65 expression and (C) fluorescence microscopy of NF-κB p65 nuclear translocation in LPS-induced BV-2 cells treated with or without EFSC (100, 200 and 400 mg/mL) for 2 h. Red fluorescence represents the NF-κB p65 subunit, and blue fluorescence represents nuclear DAPI staining (bar = 50 μm). The arrows indicate specific cells. *P<0.01, *vs*. control group, △P<0.01, *vs*. LPS group

neurons and conditional medium, EFSC could resist various damage agents released from activated BV-2 cells and exert neuronprotection effect.

Evidence implicated NF-κB may play key regulatory roles in the transcription of pro-inflammatory mediators and cytokines in the pathogenesis of

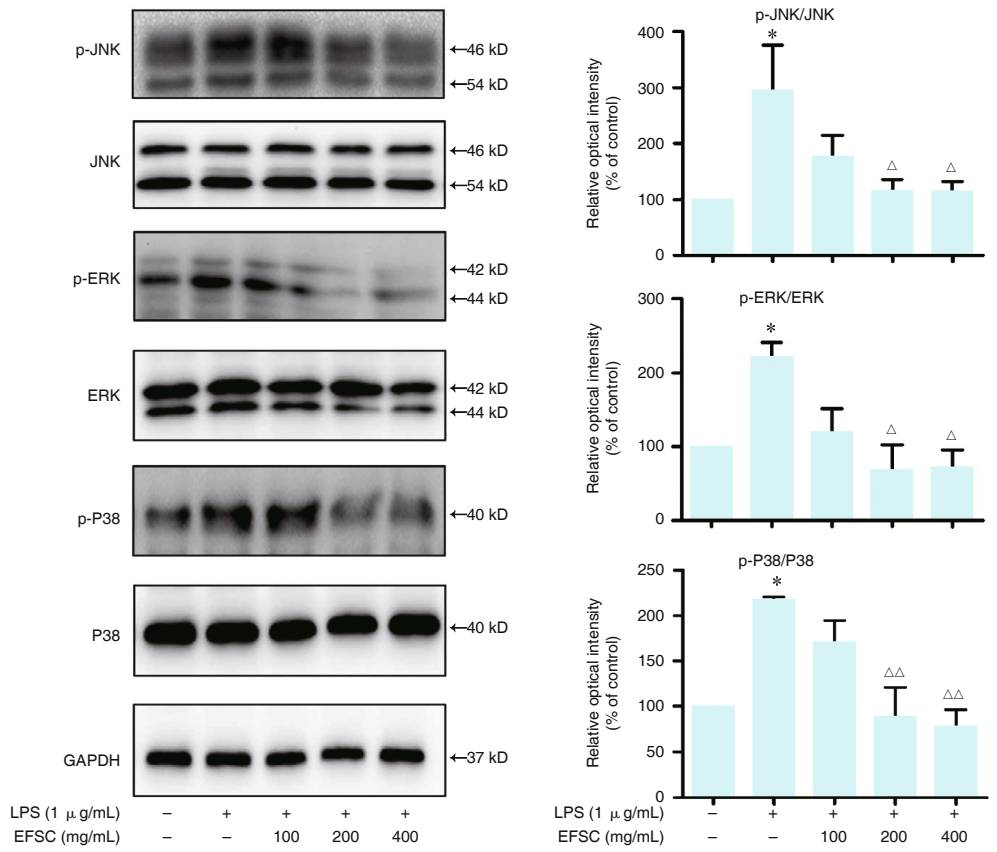

Figure 5. EFSC Inhibits Activation of MAPKs Pathway in LPS-Induced BV-2 Cells

Notes: Western blot analysis for the expressions of p-JNK, p-P38 and p-ERK in LPS-induced BV-2 cells treated with or without EFSC (100, 200 and 400 mg/mL) for 1 h. $^{*}P<0.05$, *vs*. control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. LPS group.

inflammatory diseases. In un-stimulated cells, NF-κB is sequestered in the cytoplasm and its nuclear localization signals was masked by IκBs (Inhibitor of κB).[25] When cells were activated by stimulating factors, IκBs were degraded by IκB kinase and free NF-κB. Then NF-κB translocates into the nucleus and specifically binds to target DNA elements, followed by activating the transcription of genes which encode inflammation proteins and factors.[26,27] The inhibition of the NF-κB may explain the potent activity of EFSC as a suppressor of inflammatory response. The present study indicated EFSC could reverse NF-κB p65 translocation from the cytoplasm to the nucleus when treated with LPS. These effects might arise through suppression of activation and phosphorylation NF-κB.

Besides NF-κB pathway, mounting evidence have suggested that MAPKs may play a vital role in mediating important biological processes and cellular responses to inflammatory stimulation.[28,29] The MAPK signaling pathway is also regarded as a key molecular target for the research of potential anti-inflammatory drugs. ERK MAPKs are matter to cell survival, whereas JNKs and p38-MAPKs were deemed stress responsive and thus involved in apoptosis. In this study, we investigated whether EFSC exerted an inhibitory effect on the phosphorylation level of JNK, ERK and p38 in LPS-induced BV-2 cells. Based on our study, EFSC could significantly suppress the increased phosphorylation level of the three MAPKs and indicated that suppression of the MAPK signal pathway might be involved in the anti-inflammatory effects.

In conclusion, our findings indicated that EFSC acted as an anti-inflammatory agent in LPS-induced glia cells. These effects might be realized through blocking of NF-κB activity and inhibition of the MAPKs signaling pathway. EFSC supplementation may be useful for preventing neuroinflammatory diseases.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

Wang XM , Zeng KW and Song FJ designed the research; Song FJ, Chen JF, LI Y and Song XM performed the research;

Tu PF and Zeng KW contributed the reagents, materials and analysis tools; Song FJ, Chen JF and Zeng KW analyzed the data; and Song FJ and Chen JF wrote the paper.

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